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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent of: Manuel Baca *et al.* -- § 156

Docket No: 22338-80060

Patent No.: 6,884,879

Assignee: Genentech, Inc.

Issued: April 26, 2005

Unit: OPLA

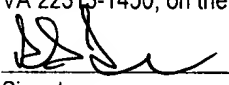
Application No: 08/908,469

For: ANTI-VEGF ANTIBODIES – Application for  
§ 156 Patent Term Extension

CERTIFICATE OF MAILING - 37 C.F.R. § 1.10  
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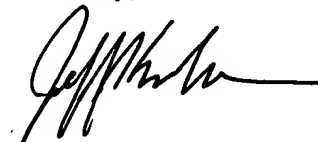
 David Deamon Aug. 25, 2006  
Signature Printed Name Date

APPLICATION FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. § 156

Dear Sir:

Pursuant to 37 C.F.R. § 1.741(c) Applicant, Genentech, Inc., today filed three (3) copies of the Application for § 156 Patent Term Extension of U.S. Patent No. 6,884,879. Pursuant to MPEP § 2753 Applicant is submitting herewith two (2) additional copies of this Application.

Sincerely,



Jeffrey P. Kushan  
Attorney for Applicant  
Registration No. 43,401

Sidley Austin LLP  
1501 K Street, N.W.  
Washington, D.C. 20005

Dated: August 25, 2006



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent of: Manuel Baca *et al.* -- § 156

Patent No.: 6,884,879

Issued: April 26, 2005

Application No: 08/908,469

For: ANTI-VEGF ANTIBODIES – Application for  
§ 156 Patent Term Extension

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EXPRESS MAIL LABEL NO. ER 736 919 908 US

I hereby certify this correspondence is being deposited with the U.S. Postal Service with sufficient postage as "Express Mail – Post Office to Addressee" addressed to: Mail Stop Patent Ext., Commissioner for Patents, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

[Signature] YVONNE T. REYES Aug 25 2006  
Signature Printed Name Date

APPLICATION FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. § 156

Dear Sir:

Applicant, Genentech, Inc., hereby submits this application for extension of the term of United States Letters Patent 6,884,879 under 35 U.S.C. § 156 by providing the following information in accordance with the requirements specified in 37 C.F.R. § 1.740.

Applicant represents that it is the assignee of the entire interest in and to United States Letters Patent No. 6,884,879, granted to Manuel Baca; James A. Wells; Leonard G. Presta; Henry B. Lowman; and Yvonne Man-yee Chen (Baca *et al.*) by virtue of an assignment of such patent to Genentech, Inc., recorded December 29, 1997, at Reel 8872, Frame 0429.

1. Identification of the Approved Product [§ 1.740(a)(1)]

The name of the approved product is LUCENTIS™. The name of the active ingredient of LUCENTIS™ is ranibizumab. Ranibizumab is a recombinant humanized monoclonal IgG<sub>1</sub> antibody antigen-binding fragment (Fab) based on a humanized framework with complementarity-determining regions (CDRs) derived from a murine monoclonal antibody that binds to human Vascular Endothelial Growth Factor (VEGF).

**2. Federal Statute Governing Regulatory Approval of the Approved Product  
[§ 1.740(a)(2)]**

The approved product was subject to regulatory review under, *inter alia*, the Public Health Service Act (42 U.S.C. § 201 *et seq.*) and the Federal Food, Drug and Cosmetic Act (21 U.S.C. § 355 *et seq.*).

**3. Date of Approval for Commercial Marketing [§ 1.740(a)(3)]**

LUCENTIS™ was approved for commercial marketing or use under § 351 of the Public Health Service Act on **June 30, 2006**.

**4. Identification of Active Ingredient and Certifications Related to Commercial Marketing of Approved Product [§ 1.740(a)(4)]**

- (a) The active ingredient of LUCENTIS™ is ranibizumab. Ranibizumab is a humanized monoclonal IgG<sub>1</sub> antibody antigen-binding fragment produced by an *E. coli* expression system. It contains human framework regions (FRs) and the complementarity-determining regions (CDRs) derived from a murine antibody that binds to VEGF.
- (b) Applicant certifies that ranibizumab had not been approved for commercial marketing or use under the Federal Food, Drug and Cosmetic Act, the Public Health Service Act or the Virus-Serum-Toxin Act prior to the approval granted on June 30, 2006 to the present Applicant.
- (c) Ranibizumab has been approved for the treatment of patients with neovascular (wet) age-related macular degeneration. See LUCENTIS™ product label, provided as Attachment A.
- (d) LUCENTIS™ was approved for commercial marketing pursuant to § 351 of the Public Health Service Act (42 U.S.C. § 262) under Genentech's existing Department of Health and Human Services (DHHS) U.S. License No. 1048. See LUCENTIS™ approval letter, provided as Attachment B.

**5. Statement Regarding Timeliness of Submission of Patent Term Extension Request [§ 1.740(a)(5)]**

Applicant certifies that this application for patent term extension is being timely submitted within the sixty (60) day period permitted for submission specified in 35 U.S.C. § 156(d)(1) and 37 C.F.R. § 1.720(f). The last date on which this application may be submitted is August 28, 2006.

**6. Complete Identification of the Patent for Which Extension Is Being Sought  
[§ 1.740(a)(6)]**

The complete identification of the patent for which an extension is being sought is as follows:

- (a) Names of the inventors: Manuel Baca; James A. Wells; Leonard G. Presta; Henry B. Lowman; and Yvonne Man-yee Chen.
- (b) Patent Number: 6,884,879 (“the ‘879 patent”)
- (c) Date of Issue: April 26, 2005
- (d) Date of Expiration: August 6, 2017<sup>1</sup>

**7. Copy of the Patent for Which an Extension is Being Sought [§ 1.740(a)(7)]**

A copy of U.S. Patent No. 6,884,879 is provided as Attachment D to the present application.

**8. Copies of Disclaimers, Certificates of Correction, Receipt of Maintenance Fee Payment, or Reexamination Certificate [§ 1.740(a)(8)]**

- (a) U.S. Patent No. 6,884,879 is not subject to a terminal disclaimer.
- (b) A Certificate of Correction was issued for U.S. Patent No. 6,884,879 on August 23, 2005. A request for a second Certificate of Correction was filed on May 19, 2006. The request asks that the related application data in the patent be amended to accurately reflect that application No. 08/883,504 had been converted to provisional application no. 60/126,446. A copy of the Certificate of Correction as well as the Request for the second Certificate of Correction is provided in Attachment E to the present application.
- (c) The first maintenance fee for U.S. Patent No. 6,884,879 will be due on October 26, 2008.

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<sup>1</sup> The face sheet of the ‘879 patent notes that a claim of priority is made to U.S. Serial No. 08/833,504 (“the ‘504 application”). Prior to abandonment of that application and issuance of the ‘879 patent, a petition was filed to convert the ‘504 application to a provisional application. The petition was submitted on April 6, 1998 and granted thereafter (a copy of the PTO’s entry of the petition is being provided for convenience as Attachment C) and the ‘504 application became U.S. Provisional Application No. 60/126,446. Accordingly, the filing date of the 08/908,469 application, from which the ‘879 patent issued, is used in calculating the expiration date of the ‘879 patent.

- (d) U.S. Patent No. 6,884,879 has not been the subject of a reexamination proceeding.

**9. Statement Regarding Patent Claims Relative to Approved Product [§ 1.740(a)(9)]**

*The statements below are made solely to comply with the requirements of 37 C.F.R. § 1.740(a)(9). Applicant notes that, as the M.P.E.P. acknowledges, § 1.740(a)(9) does not require an applicant to show whether or how the listed claims would be infringed, and that this question cannot be answered without specific knowledge concerning acts performed by third parties. As such, these comments are not an assertion or an admission of Applicant as to the scope of the listed claims, or whether or how any of the listed claims would be infringed, literally or under the doctrine of equivalents, by the manufacture, use, sale, offer for sale or the importation of any product.*

- (a) At least claims 4 and 5 of U.S. Patent No. 6,884,879 claim the active pharmaceutical ingredient in the approved product or a method that may be used to make or use that ingredient.
- (b) Pursuant to M.P.E.P. § 2753 and 37 C.F.R. § 1.740(a)(9), the following explanation is provided which shows how the above-listed claims of the '879 patent claim a method of making the approved product.

*(1) Description of the approved product and its method of production*

The approved product is described in Section 11 of the approved label for LUCENTIS™ as follows, a copy of which is provided as Attachment A.

LUCENTIS™ (ranibizumab injection) is a recombinant humanized IgG1 kappa isotype monoclonal antibody fragment designed for intraocular use. Ranibizumab binds to and inhibits the biologic activity of human vascular endothelial growth factor A (VEGF-A). Ranibizumab has a molecular weight of approximately 48 kilodaltons and is produced by an *E. coli* expression system in a nutrient medium containing the antibiotic tetracycline. Tetracycline is not detectable in the final product.

LUCENTIS™ is a sterile, colorless to pale yellow solution in a single-use glass vial. LUCENTIS™ is supplied as a preservative-free, sterile solution in a single-use glass vial designed to deliver 0.05 mL of 10 mg/mL LUCENTIS™ aqueous solution with 10 mM histidine HCL, 10% α, α-trehalose dihydrate, 0.01% polysorbate 20, pH 5.5.

Ranibizumab is further characterized in a scientific reference, Chen *et al*, published in 1999 in the Journal of Molecular Biology (JMB) entitled "Selection and Analysis of an Optimized Anti-VEGF Antibody: Crystal Structure of an

Affinity-matured Fab in Complex with Antigen.”<sup>2</sup> For example, the heavy chain sequence of ranibizumab, designated as Y0317 in Chen *et al.*, is displayed in Figure 1. The CDRH1, 2, and 3 of ranibizumab are underlined in the figure and have the following sequences:

CDRH1 contains 10 amino acids of the sequence: GYDFTHYGMN (which using the three-letter designation for each amino acid is the sequence Gly Tyr Asp Phe Thr His Tyr Gly Met Asn):

CDRH2 contains 17 amino acids of the sequence: WINTYTGEPTYAADFQR (which using the three-letter designation for each amino acid is the sequence Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe Lys Arg); and

CDRH3 contains 14 amino acids of the sequence: YPYYGTSHWYFDV (which using the three-letter designation for each amino acid is the sequence Tyr Pro Tyr Tyr Tyr Gly Thr Ser His Trp Tyr Phe Asp Val)

The article also discusses the lineage of the ranibizumab antibody fragment. In that respect, the article states that “[a] murine monoclonal antibody, A.4.6.1, was found to block VEGF-dependant cell proliferation *in vitro* and to antagonize tumor growth *in vivo*. [Citation omitted]. The murine mAb was previously humanized in Fab form to yield a variant known as Fab-12.” [Citation omitted] See p. 866, left col., ¶1. The abstract explains that the authors affinity-matured Fab-12 and obtained Fab fragment Y0317, now known as ranibizumab. According to the article, ranibizumab was derived from the humanization and affinity-maturation of a non-human, murine monoclonal antibody that binds to VEGF.

(2) *Claim 4 and 5*

Claim 4 of the '879 patent reads as follows:

4. A process of producing a humanized anti-VEGF antibody comprising culturing the host cell of claim 3 so that the nucleic acid is expressed.
5. The process of claim 4 further comprising recovering the humanized anti-VEGF antibody from the host cell culture.

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<sup>2</sup> 293:865-881 (1999) (Attachment F)

Claim 4 depends from claim 3, which in turn depends from claim 2, which depends from claim 1. Therefore, claim 4 contains the limitations of claims 1, 2, and 3. Claim 5 further depends from claim 4. Claims 1, 2, and 3 of the '879 patent read as follows:

1. Isolated nucleic acid encoding a humanized variant of a parent anti-VEGF antibody which parent antibody comprises non-human variable domains, wherein said humanized variant binds human VEGF and comprises the following heavy chain Complementary Determining Region (CDR) amino acid sequences: SEQ ID NO:128 as CDRH1, SEQ ID NO:2 as CDRH2 and SEQ ID NO:129 as CDRH3.
2. A vector comprising the nucleic acid of claim 1.
3. An isolated host cell comprising the vector of claim 2.

*Comparison of a nucleic acid encoding ranibizumab to the limitations of claim 1*

Claim 1 pertains to an isolated nucleic acid encoding a humanized variant of a parent anti-VEGF antibody, which parent antibody comprises non-human variable domains, wherein said humanized variant binds human VEGF and comprises the recited CDRs.

As discuss above, ranibizumab was initially produced by the humanization and affinity-maturation of a murine, anti-VEGF monoclonal antibody and Fab fragment. The parental murine monoclonal antibody and its humanized fragment, by definition, contain non-human variable domains. Applicant also notes that the term "antibody" as defined in the '879 patent specification includes, in addition to full-length antibodies, antibody fragments such as Fab, Fab', F(ab)<sub>2</sub> and Fv so long as those fragments retain the desired biological activity, i.e., binding to VEGF (*See, e.g.*, Col 8, lines 38-49). Ranibizumab, being an Fab fragment that binds VEGF, falls within the scope of the term antibody as used in Claim 1. Moreover, as demonstrated by the following comparison, the CDR sequences disclosed in SEQ ID NOS:128, 2 and 129 of the '879 patent are identical to, or read on, the CDR sequence of ranibizumab disclosed in the Chen *et al* reference describe above.

SEQ ID NO:128 (CDRH1)	Gly Tyr Xaa <sup>3</sup> Phe Thr Xaa Tyr Gly Met Asn
Ranibizumab CDRH1 (Chen <i>et al.</i> )	Gly Tyr Asp Phe Thr His Tyr Gly Met Asn
SEQ ID NO:2 (CDRH2)	Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe Lys Arg
Ranibizumab CDRH2 (Chen <i>et al.</i> )	Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe Lys Arg
SEQ ID NO:129 (CDRH3)	Tyr Pro Xaa <sup>4</sup> Tyr Tyr Gly Xaa Ser His Trp Tyr Phe Asp Val
Ranibizumab CDRH3 (Chen <i>et al.</i> )	Tyr Pro Tyr Tyr Tyr Gly Thr Ser His Trp Tyr Phe Asp Val

The CDR sequences recited in claim 1 of the '879 patent thus read on the CDR sequences of ranibizumab.

*Comparison of the production of ranibizumab to the limitations of claim 2, 3 and 4*

Claim 2 and 3 of the '879 patent recite a vector containing the nucleic acid of claim 1 and an isolated host cell containing said vector, respectively. Claim 4 additionally requires a method of producing an anti-VEGF antibody. Pursuant to the label, ranibizumab is produced using a recombinant *E. coli* cell line expression system. Such an expression system employs a *vector*, which as explained above would contain a nucleic acid covered by claim 1, and an isolated host cell, in this case *E. coli*, containing the vector. Using the vector of claim 2 in an *E. coli* host cell of claim 3 as a method of producing ranibizumab, would fall within the scope of claim 4 of the '879 patent.

*Comparison of the production of ranibizumab to Claim 5*

Claim 5 pertains to a process wherein the humanized anti-VEGF antibody is recovered from the host cell culture used to make the antibody. Applicant asserts that claim 5 covers a method of making the approved product for at least the following reasons.

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<sup>3</sup> SEQ ID NO:128 notes that the third amino acid can be either Thr or Asp. The sixth amino acid in SEQ ID NO:128, on the other hand, is not limited to any particular amino acid.

<sup>4</sup> SEQ ID NO:129 notes that the third amino acid can be either Tyr or His. Similarly, it notes that the seventh amino acid can be either Ser or Thr.



According to the label, Lucentis™ contains ranibizumab in a form that is sterile and suitable for intraocular injection. *See* Label ¶11. The label also states that ranibizumab is produced using an *E. coli* expression system. While the label does not provide the details of how ranibizumab is purified for use in Lucentis™, the antibody would necessarily have to be separated and recovered from the cell culture in which it was produced in order for it to be used in a sterile, injectible therapeutic. Accordingly, the purification steps used in the manufacture of ranibizumab are covered by claim 5.

**10. Relevant Dates Under 35 U.S.C. § 156 for Determination of Applicable Regulatory Review Period [§ 1.740(a)(10)]**

*(a) Patent Issue Date*

U.S. Patent No. 6,884,879 was issued on April 26, 2005.

*(b) IND Effective Date [35 U.S.C. § 156(g)(1)(B)(i); 37 C.F.R. § 1.740(a)(10)(i)(A)]*

The date that an exemption under § 505(i) of the Federal Food, Drug and Cosmetic Act became effective (*i.e.*, the date that an investigational new drug application (“IND”) became effective) for LUCENTIS™ (referred to as “Humanized Monoclonal Antibody Fragment (rhuFab V2)(E. coli, Genentech) to Vascular Endothelial Growth Factor (VEGF), Intravitreal”) was October 7, 1999. The IND was assigned number BB-IND # 8633. A copy of the letter from the FDA reflecting the effective date of the IND is provided in Attachment G. The application date for this IND was October 6, 1999.

*(c) BLA Submission Date [35 U.S.C. § 156(g)(1)(B)(i); 37 C.F.R. § 1.740(a)(10)(i)(B)]*

The BLA was submitted by Genentech to the FDA on December 29, 2005. The BLA was assigned number BL# 125156/0. A copy of the letter from the FDA acknowledging receipt of the BLA and reflecting the BLA submission date is provided in Attachment H.

*(d) BLA Issue Date [35 U.S.C. § 156(g)(1)(B)(ii); 37 C.F.R. § 1.740(a)(10)(i)(C)]*

The FDA approved biologic license application 125156/0 authorizing the marketing of LUCENTIS™ on June 30, 2006. LUCENTIS™ was approved under Department of Health and Human Services (DHHS) U.S. License No. 1048. A copy of the approval letter from the FDA is provided as Attachment B.

**11. Summary of Significant Events During Regulatory Review Period [§ 1.740(a)(11)]**

Pursuant to 37 C.F.R. § 1.740(a)(11), the following provides a brief description of the activities of Genentech, Inc. before the FDA in relation to the regulatory review of LUCENTIS™. The brief description lists the significant events that occurred during the regulatory review period for the approved product. In several instances, communications to or from the FDA are referenced. Pursuant to 37 C.F.R. § 1.740(a)(11), 21 C.F.R. § 60.20(a), and M.P.E.P. § 2753, copies of all such communications are not provided in this application, but can be obtained from records maintained by the FDA.

- On October 6, 1999, Genentech submitted to FDA (*See Attachment G*) an investigational new drug application for a recombinant humanized monoclonal antibody fragment (rhuFab V2, now known as Ranibizumab) against Vascular Endothelial Growth Factor (VEGF). The antibody was developed as a potential new therapeutic in treating patients with the exudative (wet or neovascular) form of age-related macular degeneration (AMD).
- On October 7, 1999 FDA made BB-IND #8633 effective via a communication mailed to Genentech on October 13, 1999 (*See Attachment G*). According to the FDA, initiation of trials could begin 30 days after October 7, 1999.
- The first human clinical trial (Phase I) was initiated on February 8, 2000 followed by Phase II human trials and Phase III human trials, some of which remain ongoing at the time of this application.
- On February 5, 2002, representatives of Genentech and the FDA (CBER and CDER) participated in a Type C meeting to discuss the proposed clinical development plan for ranibizumab in AMD.
- On October 31, 2002 representatives of Genentech and FDA (CBER and CDER) participated in an Type B End-of-Phase II meeting.
- Beginning in approximately March 2003, and continuing at the time of this application, Phase III studies have been conducted. The three Phase III trials forming the basis of the Biologics License Application (BLA), FVF2598g, FVF2587g, and FVF3192g are studies of two year duration with primary endpoints of one year. FVF2587g and FVF3192g, along with extension study FVF3426g and safety study FVF3689g, remain ongoing at the time of this application.
- On September 21, 2005 representatives of Genentech and CDER participated in a Type B Pre-BLA submission meeting to discuss information requirements for the BLA.

- Genentech submitted a BLA for ranibizumab for the treatment of patients with wet AMD on December 29, 2005 (*See Attachment H*).
- FDA acknowledged receipt of the BLA for ranibizumab via a communication mailed to Genentech dated January 27, 2006. The letter indicated that FDA had assigned the Submission Tracking Number (STN) of BL #125156/0 to the BLA (*See Attachment H*).
- By way of a communication mailed to Genentech on March 14, 2006 FDA made Genentech aware that the BLA for ranibizumab was filed on February 28, 2006 and that FDA had assigned a user fee goal date of June 30, 2006 (*See Attachment I*).
- On June 30, 2006 FDA approved BLA 125156/0, issuing marketing authorization for LUCENTIS™ (*See Attachment B*).

**12. Statement Concerning Eligibility for and Duration of Extension Sought Under 35 U.S.C. § 156 [37 C.F.R. § 1.740(a)(12)]**

- (a) In the opinion of the Applicant, U.S. Patent No. 6,884,879 is eligible for an extension under § 156 because:
  - (i) one or more claims of the '879 patent claim the approved product or a method of making or using the approved product;
  - (ii) the term of the '879 patent has not been previously extended on the basis of § 156;
  - (iii) the '879 patent has not expired;
  - (iv) no other patent has been extended pursuant to § 156 on the basis of the regulatory review process associated with the approved product, LUCENTIS™;
  - (v) there is an eligible period of regulatory review by which the patent may be extended pursuant to § 156;
  - (vi) the applicant for marketing approval exercised due diligence within the meaning of § 156(d)(3) during the period of regulatory review;
  - (vii) the present application has been submitted within the 60-day period following the approval date of the approved product, pursuant to § 156(c); and
  - (viii) this application otherwise complies with all requirements of 35 U.S.C. § 156 and applicable rules and procedures.
- (b) The period by which the term of the '879 patent is requested by Applicant to be extended is **307 days**.
- (c) The requested period of extension of term for the '879 patent corresponds to the regulatory review period that is eligible for extension pursuant to § 156, based on the facts and circumstances of the regulatory review associated with the approved product LUCENTIS™ and the issuance of the '879 patent. The period was determined as follows.
  - (i) The relevant dates for calculating the regulatory review period, based on the events discussed in the section above, are the following.

Exemption under FDCA § 505(i) became effective	October 7, 1999
Patent was granted	April 26, 2005
Biologics License Application (BLA) under PHSA § 351 was filed	December 29, 2005
BLA was approved	June 30, 2006

- (ii) The '879 patent was granted during the period specified in § 156(g)(1)(B)(i) (*i.e.*, the period from the date of the grant of the exemption under § 505(i) of the FDCA until the date of submission of the BLA). Pursuant to § 156(b) and (c)(2), the calculated regulatory review period therefore includes a component of time between when the patent was granted and when the BLA was submitted (1/2 of 246 days or 123 days).
- (iii) The patent was granted prior to the start of the period specified in § 156(g)(1)(B)(ii) (*i.e.*, the period from the date of submission of the BLA until the date of approval). The regulatory review period under § 156(b) therefore includes a component equal to the total number of days in that period that are after the BLA was submitted (184 days).
- (iv) The period determined according to § 156(b), (c)(2), and (g)(1) for the approved product (*i.e.*, the number of days following the date of issue of the patent between the dates of submission and of approval of the BLA for LUCENTIS™) is 307 days.
- (v) The '879 patent will expire on August 6, 2017.
- (vi) The date of approval of the approved product is June 30, 2006.
- (vii) The date that is fourteen years from the date of approval of the approved product is June 30, 2020.
- (viii) The period measured from the date the patent expires (*i.e.*, August 6, 2017) until the end of the fourteen-year period specified in § 156 (c)(3) (*i.e.*, June 30, 2020) is approximately 1054 days (*ca.* 2 years, 10 months and 24 days).

- (ix) The number of days in the regulatory review period determined pursuant to § 156(g)(1)(B)(ii) does not exceed the number of days that the patent may be extended pursuant to §156(c)(3). As such, the period by which the patent may be extended is not limited by the fourteen-year rule of §156(c)(3).
- (x) The '879 patent issued after the effective date of Public Law No. 98-417. As such, the two- or three-year limit of 35 U.S.C. § 156(g)(6)(C) does not apply.

**13. Statement Pursuant to 37 C.F.R. § 1.740(a)(13)**

Pursuant to 37 C.F.R. § 1.740(a)(13), Applicant acknowledges its duty to disclose to the Director of the PTO and to the Secretary of Health and Human Services any information which is material to the determination of entitlement to the extension sought, particularly as that duty is defined in 37 C.F.R. § 1.765.

**14. Applicable Fee [§ 1.740(a)(14)]**

Our check in payment of the fee prescribed in 37 C.F.R. § 1.20(j) for a patent term extension application under 35 U.S.C. § 156 accompanies this application. Please deduct any additional required fees from, or credit any overpayments to our deposit account no. 18-1260.

**15. Name and Address for Correspondence [§ 1.740(a)(14)]**

Please direct all inquiries, questions, and communications regarding this application for term extension to:

Jeffrey P. Kushan  
SIDLEY AUSTIN LLP  
1501 K Street, N.W.  
Washington, D.C. 20005  
Phone: 202-736-8914  
Fax: 202-736-8111  
email: jkushan@sidley.com

The correspondence address for U.S. Patent No. 6,884,879 is unchanged for all other purposes. A Power of Attorney granted to the undersigned by the patent assignee, a copy of which is included with this application as Attachment J, accompanies this communication.

U.S. Patent No. 6,884,879  
Baca, *et al.*  
Application Under 35 U.S.C. § 156

Page 15

Two additional copies of this application are enclosed, in compliance with 37 C.F.R.  
§ 1.740(b).

Sincerely,

A handwritten signature in black ink, appearing to read "Jeffrey P. Kushan", with a long horizontal flourish extending to the right.

Jeffrey P. Kushan  
Attorney for Applicant  
Registration No. 43,401

Sidley Austin LLP  
1501 K Street, N.W.  
Washington, D.C. 20005

Dated: August 24, 2006



## INDEX OF ATTACHMENTS

- Attachment A: Lucentis™ Product Label
- Attachment B: Lucentis™ Biologics' License Application Approval
- Attachment C: 05/13/99 PTO petition grant
- Attachment D: U.S. Patent No. 6,884,879
- Attachment E: Certificate of Correction and request for a second Certificate of Correction
- Attachment F: Chen *et al.*, "Selection and Analysis of an Optimized Anti-VEGF Antibody: Crystal Structure of an Affinity-Matured Fab in Complex with Antigen." *J. Mol. Bio.*, 293:865-881 (1999).
- Attachment G: 10/13/99 Letter from FDA to Genentech regarding IND acceptance/effective date
- Attachment H: 01/27/06 Letter from the FDA to Genentech regarding receipt and acceptance of BLA Application
- Attachment I: 03/14/06 Letter from the FDA to Genentech regarding 02/28/06 filing of BLA, and 06/30/06 assignment of User Fee Goal Date
- Attachment J: Power of Attorney by Assignee

## HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use LUCENTIS safely and effectively. See full prescribing information for LUCENTIS.

**LUCENTIS™ (ranibizumab injection)**

**Initial U.S. Approval: 2006**

### -----INDICATIONS AND USAGE-----

LUCENTIS is indicated for the treatment of patients with neovascular (wet) age-related macular degeneration (1).

### -----DOSAGE AND ADMINISTRATION-----

- **FOR OPHTHALMIC INTRAVITREAL INJECTION ONLY (2.1)**
- LUCENTIS 0.5 mg (0.05 mL) is recommended to be administered by intravitreal injection once a month (2.2).
- Although less effective, treatment may be reduced to one injection every three months after the first four injections if monthly injections are not feasible. Compared to continued monthly dosing, dosing every 3 months will lead to an approximate 5-letter (1-line) loss of visual acuity benefit, on average, over the following 9 months. Patients should be evaluated regularly (2.2).

### -----DOSAGE FORMS AND STRENGTHS-----

- 10 mg/mL single-use vial (3)

### -----CONTRAINDICATIONS-----

- Ocular or periocular infections (4.1)
- Hypersensitivity (4.2)

### -----WARNINGS AND PRECAUTIONS-----

- Endophthalmitis and retinal detachments may occur following intravitreal injections. Patients should be monitored during the week following the injection (5.1).
- Increases in intraocular pressure have been noted within 60 minutes of intravitreal injection (5.2).

### -----ADVERSE REACTIONS-----

The most common adverse reactions (reported  $\geq 6\%$  higher in LUCENTIS-treated subjects than control subjects) are conjunctival hemorrhage, eye pain, vitreous floaters, increased intraocular pressure, and intraocular inflammation (6.2).

**To report SUSPECTED ADVERSE REACTIONS, contact Genentech at 1-888-835-2555 or FDA at 1-800-FDA-1088 or [www.fda.gov/medwatch](http://www.fda.gov/medwatch).**

See Section 17 for PATIENT COUNSELING INFORMATION.

## FULL PRESCRIBING INFORMATION: CONTENTS\*

1	INDICATIONS AND USAGE
2	DOSAGE AND ADMINISTRATION
2.1	General Dosing Information
2.2	Dosing
2.3	Preparation for Administration
2.4	Administration
2.5	Stability and Storage
3	DOSAGE FORMS AND STRENGTHS
4	CONTRAINDICATIONS
4.1	Ocular or Periocular Infections
4.2	Hypersensitivity
5	WARNINGS AND PRECAUTIONS
5.1	Endophthalmitis and Retinal Detachments
5.2	Increases in Intraocular Pressure
5.3	Thromboembolic Events
6	ADVERSE REACTIONS
6.1	Injection Procedure
6.2	Clinical Studies Experience – Ocular Events
6.3	Clinical Studies Experience – Non-Ocular Events
6.4	Immunogenicity
7	DRUG INTERACTIONS
8	USE IN SPECIFIC POPULATIONS

8.1	Pregnancy
8.3	Nursing Mothers
8.4	Pediatric Use
8.5	Geriatric Use
8.6	Patients with Renal Impairment
8.7	Patients with Hepatic Dysfunction
10	OVERDOSAGE
11	DESCRIPTION
12	CLINICAL PHARMACOLOGY
12.1	Mechanism of Action
12.2	Pharmacodynamics
12.3	Pharmacokinetics
13	NONCLINICAL TOXICOLOGY
13.1	Carcinogenesis, Mutagenesis, Impairment of Fertility
14	CLINICAL STUDIES
14.1	Study 1 and Study 2
14.2	Study 3
16	HOW SUPPLIED/STORAGE AND HANDLING
17	PATIENT COUNSELING INFORMATION

\* Sections or subsections omitted from the Full Prescribing Information are not listed.

U.S. BLA (BL125156) Ranibizumab injection

Genentech, Inc.

## FULL PRESCRIBING INFORMATION

### 1 INDICATIONS AND USAGE

LUCENTIS is indicated for the treatment of patients with neovascular (wet) age-related macular degeneration.

### 2 DOSAGE AND ADMINISTRATION

#### 2.1 General Dosing Information

FOR OPHTHALMIC INTRAVITREAL INJECTION ONLY.

#### 2.2 Dosing

LUCENTIS 0.5 mg (0.05 mL) is recommended to be administered by intravitreal injection once a month.

Although less effective, treatment may be reduced to one injection every three months after the first four injections if monthly injections are not feasible. Compared to continued monthly dosing, dosing every 3 months will lead to an approximate 5-letter (1-line) loss of visual acuity benefit, on average, over the following 9 months. Patients should be evaluated regularly [*see Clinical Studies (14.2)*].

#### 2.3 Preparation for Administration

Using aseptic technique, all (0.2 mL) of the LUCENTIS vial contents are withdrawn through a 5-micron 19-gauge filter needle attached to a 1-cc tuberculin syringe. The filter needle should be discarded after withdrawal of the vial contents and should not be used for intravitreal injection. The filter needle should be replaced with a sterile 30-gauge × 1/2-inch needle for the intravitreal injection. The contents should be expelled until the plunger tip is aligned with the line that marks 0.05 mL on the syringe.

#### 2.4 Administration

The intravitreal injection procedure should be carried out under controlled aseptic conditions, which include the use of sterile gloves, a sterile drape, and a sterile eyelid speculum (or equivalent). Adequate anesthesia and a broad-spectrum microbicide should be given prior to the injection.

Following the intravitreal injection, patients should be monitored for elevation in intraocular pressure and for endophthalmitis. Monitoring may consist of a check for perfusion of the optic nerve head immediately after the injection, tonometry within 30 minutes following the injection, and biomicroscopy between two and seven days following the injection. Patients should be instructed to report any symptoms suggestive of endophthalmitis without delay.

Each vial should only be used for the treatment of a single eye. If the contralateral eye requires treatment, a new vial should be used and the sterile field, syringe, gloves, drapes, eyelid speculum, filter, and injection needles should be changed before LUCENTIS is administered to the other eye.

No special dosage modification is required for any of the populations that have been studied (e.g., gender, elderly).

### 2.5 Stability and Storage

LUCENTIS should be refrigerated at 2°-8°C (36°-46°F). DO NOT FREEZE. Do not use beyond the date stamped on the label. LUCENTIS vials should be protected from light. Store in the original carton until time of use.

### 3 DOSAGE FORMS AND STRENGTHS

Single-use glass vial designed to deliver 0.05 mL of 10 mg/mL.

### 4 CONTRAINDICATIONS

#### 4.1 Ocular or Periocular Infections

LUCENTIS is contraindicated in patients with ocular or periocular infections.

#### 4.2 Hypersensitivity

LUCENTIS is contraindicated in patients with known hypersensitivity to ranibizumab or any of the excipients in LUCENTIS.

### 5 WARNINGS AND PRECAUTIONS

#### 5.1 Endophthalmitis and Retinal Detachments

Intravitreal injections, including those with LUCENTIS, have been associated with endophthalmitis and retinal detachments. Proper aseptic injection technique should always be used when administering LUCENTIS. In addition, patients should be monitored during the week following the injection to permit early treatment should an infection occur [*see Dosage and Administration (2.3, 2.4) and Patient Counseling Information (17)*].

#### 5.2 Increases in Intraocular Pressure

Increases in intraocular pressure have been noted within 60 minutes of intravitreal injection with LUCENTIS. Therefore, intraocular pressure as well as the perfusion of the optic nerve head should be monitored and managed appropriately [*see Dosage and Administration (2.4)*].

#### 5.3 Thromboembolic Events

Although there was a low rate (<4%) of arterial thromboembolic events observed in the LUCENTIS clinical trials, there is a theoretical risk of arterial thromboembolic events following intravitreal use of inhibitors of VEGF [*see Adverse Reactions (6.3)*].

### 6 ADVERSE REACTIONS

#### 6.1 Injection Procedure

Serious adverse events related to the injection procedure have occurred in <0.1% of intravitreal injections, including endophthalmitis [*see Warnings and Precautions (5.1)*], rhegmatogenous retinal detachments, and iatrogenic traumatic cataracts.

#### 6.2 Clinical Trials Experience – Ocular Events

Other serious ocular adverse events observed among LUCENTIS-treated patients occurring in <2% of patients

included intraocular inflammation and increased intraocular pressure [see *Warnings and Precautions* (5.1, 5.2)].

The available safety data include exposure to LUCENTIS in 874 patients with neovascular age-related macular degeneration in three double-masked, controlled studies with dosage regimens of 0.3 mg (375 patients) or 0.5 mg (379 patients) administered monthly by intravitreal injection (Studies 1 and 2) [see *Clinical Studies* (14.1)] and dosage regimens of 0.3 mg (59 patients) or 0.5 mg (61 patients) administered once a month for 3 consecutive doses followed by a dose administered once every 3 months (Study 3) [see *Clinical Studies* (14.2)].

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in one clinical trial of a drug cannot be directly compared with rates in the clinical trials of the same or another drug and may not reflect the rates observed in practice.

Table 1 shows the most frequently reported ocular adverse events that were reported with LUCENTIS treatment. The ranges represent the maximum and minimum rates across all three studies for control, and across all three studies and both dose groups for LUCENTIS.

**Table 1**

Adverse Event	LUCENTIS	Control
Conjunctival hemorrhage	77%-43%	66%-29%
Eye pain	37%-17%	33%-11%
Vitreous floaters	32%-3%	10%-3%
Retinal hemorrhage	26%-15%	56%-37%
Intraocular pressure increased	24%-8%	7%-3%
Vitreous detachment	22%-7%	18%-13%
Intraocular inflammation	18%-5%	11%-3%
Eye irritation	19%-4%	20%-6%
Cataract	16%-5%	16%-6%
Foreign body sensation in eyes	19%-6%	14%-6%
Lacrimation increased	17%-3%	16%-0%
Eye pruritis	13%-0%	12%-3%
Visual disturbance	14%-0%	9%-2%
Blepharitis	13%-3%	9%-4%
Subretinal fibrosis	13%-0%	19%-10%
Ocular hyperemia	10%-5%	10%-1%
Maculopathy	10%-3%	11%-3%
Visual acuity blurred/decreased	17%-4%	24%-10%
Detachment of the retinal pigment epithelium	11%-1%	15%-3%
Dry eye	10%-3%	8%-5%
Ocular discomfort	8%-0%	5%-0%
Conjunctival hyperemia	9%-0%	7%-0%
Posterior capsule opacification	8%-0%	5%-0%
Retinal exudates	9%-1%	11%-3%

### 6.3 Clinical Trials Experience – Non-Ocular Events

Table 2 shows the most frequently reported non-ocular adverse events with LUCENTIS treatment. The ranges represent the maximum and minimum rates across all three studies for control, and across all three studies and both dose groups for LUCENTIS.

**Table 2**

Adverse Event	LUCENTIS	Control
Hypertension/elevated blood pressure	23%-5%	23%-8%
Nasopharyngitis	16%-5%	13%-5%
Arthralgia	11%-3%	9%-0%
Headache	15%-2%	10%-3%
Bronchitis	10%-3%	8%-2%
Cough	10%-3%	7%-2%
Anemia	8%-3%	8%-0%
Nausea	9%-2%	6%-4%
Sinusitis	8%-2%	6%-4%
Upper respiratory tract infection	15%-2%	10%-4%
Back pain	10%-1%	9%-0%
Urinary tract infection	9%-4%	8%-5%
Influenza	10%-2%	5%-1%
Arthritis	8%-0%	8%-2%
Dizziness	8%-2%	10%-2%
Constipation	7%-3%	8%-2%

The rate of arterial thromboembolic events in the three studies in the first year was 2.1% of patients (18 out of 874) in the combined group of patients treated with 0.3 mg or 0.5 mg LUCENTIS compared with 1.1% of patients (5 out of 441) in the control arms of the studies. In the second year of Study 1, the rate of arterial thromboembolic events was 3.0% of patients (14 out of 466) in the combined group of patients treated with 0.3 mg or 0.5 mg LUCENTIS compared with 3.2% of patients (7 out of 216) in the control arm [see *Warnings and Precautions* (5.3)].

### 6.4 Immunogenicity

The pre-treatment incidence of immunoreactivity to LUCENTIS was 0%-3% across treatment groups. After monthly dosing with LUCENTIS for 12 to 24 months, low titers of antibodies to LUCENTIS were detected in approximately 1%-6% of patients. The immunogenicity data reflect the percentage of patients whose test results were considered positive for antibodies to LUCENTIS in an electrochemiluminescence assay and are highly dependent on the sensitivity and specificity of the assay. The clinical significance of immunoreactivity to LUCENTIS is unclear at this time, although some patients with the highest levels of immunoreactivity were noted to have iritis or vitritis.

### 7 DRUG INTERACTIONS

Drug interaction studies have not been conducted with LUCENTIS.

LUCENTIS intravitreal injection has been used adjunctively with verteporfin photodynamic therapy (PDT). Twelve of 105 (11%) patients developed serious intraocular inflammation; in 10 of the 12 patients, this occurred when LUCENTIS was administered 7 days ( $\pm$  2 days) after verteporfin PDT.

## 8 USE IN SPECIFIC POPULATIONS

### 8.1 Pregnancy

Pregnancy Category C. Animal reproduction studies have not been conducted with ranibizumab. It is also not known whether ranibizumab can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. LUCENTIS should be given to a pregnant woman only if clearly needed.

### 8.3 Nursing Mothers

It is not known whether ranibizumab is excreted in human milk. Because many drugs are excreted in human milk, and because the potential for absorption and harm to infant growth and development exists, caution should be exercised when LUCENTIS is administered to a nursing woman.

### 8.4 Pediatric Use

The safety and effectiveness of LUCENTIS in pediatric patients has not been established.

### 8.5 Geriatric Use

In the controlled clinical studies, approximately 94% (822/879) of the patients randomized to treatment with LUCENTIS were  $\geq$  65 years of age and approximately 68% (601/879) were  $\geq$  75 years of age. No notable difference in treatment effect was seen with increasing age in any of the studies. Age did not have a significant effect on systemic exposure in a population pharmacokinetic analysis after correcting for creatinine clearance.

### 8.6 Patients with Renal Impairment

No formal studies have been conducted to examine the pharmacokinetics of ranibizumab in patients with renal impairment. Sixty-eight percent of patients (136 of 200) in the population pharmacokinetic analysis had renal impairment (46.5% mild, 20% moderate, and 1.5% severe). Reduction in ranibizumab clearance is minimal in patients with renal impairment and is considered clinically insignificant. Dose adjustment is not expected to be needed for patients with renal impairment.

### 8.7 Patients with Hepatic Dysfunction

No formal studies have been conducted to examine the pharmacokinetics of ranibizumab in patients with hepatic impairment. Dose adjustment is not expected to be needed for patients with hepatic dysfunction.

## 10 OVERDOSAGE

Planned initial single doses of ranibizumab injection 1.0 mg were associated with clinically significant intraocular inflammation in 2 of 2 patients injected. With an escalating regimen of doses beginning with initial doses of ranibizumab

injection 0.3 mg, doses as high as 2.0 mg were tolerated in 15 of 20 patients.

## 11 DESCRIPTION

LUCENTIS™ (ranibizumab injection) is a recombinant humanized IgG1 kappa isotype monoclonal antibody fragment designed for intraocular use. Ranibizumab binds to and inhibits the biologic activity of human vascular endothelial growth factor A (VEGF-A). Ranibizumab has a molecular weight of approximately 48 kilodaltons and is produced by an *E. coli* expression system in a nutrient medium containing the antibiotic tetracycline. Tetracycline is not detectable in the final product.

LUCENTIS is a sterile, colorless to pale yellow solution in a single-use glass vial. LUCENTIS is supplied as a preservative-free, sterile solution in a single-use glass vial designed to deliver 0.05 mL of 10 mg/mL LUCENTIS aqueous solution with 10 mM histidine HCl, 10%  $\alpha$ ,  $\alpha$ -trehalose dihydrate, 0.01% polysorbate 20, pH 5.5.

## 12 CLINICAL PHARMACOLOGY

### 12.1 Mechanism of Action

Ranibizumab binds to the receptor binding site of active forms of VEGF-A, including the biologically active, cleaved form of this molecule, VEGF<sub>110</sub>. VEGF-A has been shown to cause neovascularization and leakage in models of ocular angiogenesis and is thought to contribute to the progression of the neovascular form of age-related macular degeneration (AMD). The binding of ranibizumab to VEGF-A prevents the interaction of VEGF-A with its receptors (VEGFR1 and VEGFR2) on the surface of endothelial cells, reducing endothelial cell proliferation, vascular leakage, and new blood vessel formation.

### 12.2 Pharmacodynamics

Neovascular AMD is associated with foveal retinal thickening as assessed by optical coherence tomography (OCT) and leakage from CNV as assessed by fluorescein angiography.

In Study 3, foveal retinal thickness was assessed by OCT in 118/184 patients. OCT measurements were collected at baseline, Months 1, 2, 3, 5, 8, and 12. In patients treated with LUCENTIS, foveal retinal thickness decreased, on average, more than the sham group from baseline through Month 12. Retinal thickness decreased by Month 1 and decreased further at Month 3, on average. Foveal retinal thickness data did not provide information useful in influencing treatment decisions [see *Clinical Studies* (14.2)].

In patients treated with LUCENTIS, the area of vascular leakage, on average, decreased by Month 3 as assessed by fluorescein angiography. The area of vascular leakage for an individual patient was not correlated with visual acuity.

### 12.3 Pharmacokinetics

In animal studies, following intravitreal injection, ranibizumab was cleared from the vitreous with a half-life of approximately 3 days. After reaching a maximum at approximately 1 day,

the serum concentration of ranibizumab declined in parallel with the vitreous concentration. In these animal studies, systemic exposure of ranibizumab is more than 2000-fold lower than in the vitreous.

In patients with neovascular AMD, following monthly intravitreal administration, maximum ranibizumab serum concentrations were low (0.3 ng/mL to 2.36 ng/mL). These levels were below the concentration of ranibizumab (11 ng/mL to 27 ng/mL) thought to be necessary to inhibit the biological activity of VEGF-A by 50%, as measured in an in vitro cellular proliferation assay. The maximum observed serum concentration was dose proportional over the dose range of 0.05 to 1.0 mg/eye. Based on a population pharmacokinetic analysis, maximum serum concentrations of 1.5 ng/mL are predicted to be reached at approximately 1 day after monthly intravitreal administration of LUCENTIS 0.5 mg/eye. Based on the disappearance of ranibizumab from serum, the estimated average vitreous elimination half-life was approximately 9 days. Steady-state minimum concentration is predicted to be 0.22 ng/mL with a monthly dosing regimen. In humans, serum ranibizumab concentrations are predicted to be approximately 90,000-fold lower than vitreal concentrations.

### 13 NONCLINICAL TOXICOLOGY

#### 13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

No carcinogenicity or mutagenicity data are available for ranibizumab injection in animals or humans.

No studies on the effects of ranibizumab on fertility have been conducted.

### 14 CLINICAL STUDIES

The safety and efficacy of LUCENTIS were assessed in three randomized, double-masked, sham- or active-controlled studies in patients with neovascular AMD. A total of 1323 patients (LUCENTIS 879, Control 444) were enrolled in the three studies.

#### 14.1 Study 1 and Study 2

In Study 1, patients with minimally classic or occult (without classic) CNV lesions received monthly LUCENTIS 0.3 mg or 0.5 mg intravitreal injections or monthly sham injections. Data are available through Month 24. Patients treated with LUCENTIS in Study 1 received a mean of 22 total treatments out of a possible 24 from Day 0 to Month 24.

In Study 2, patients with predominantly classic CNV lesions received one of the following: 1) monthly LUCENTIS 0.3 mg intravitreal injections and sham PDT; 2) monthly LUCENTIS 0.5 mg intravitreal injections and sham PDT; or 3) sham intravitreal injections and active verteporfin PDT. Sham PDT (or active verteporfin PDT) was given with the initial LUCENTIS (or sham) intravitreal injection and every 3 months thereafter if fluorescein angiography showed persistence or recurrence of leakage. Data are available through Month 12. Patients treated with LUCENTIS in

Study 2 received a mean of 12 total treatments out of a possible 13 from Day 0 through Month 12.

In both studies, the primary efficacy endpoint was the proportion of patients who maintained vision, defined as losing fewer than 15 letters of visual acuity at 12 months compared with baseline. Almost all LUCENTIS-treated patients (approximately 95%) maintained their visual acuity. 34%-40% of LUCENTIS-treated patients experienced a clinically significant improvement in vision, defined as gaining 15 or more letters at 12 months. The size of the lesion did not significantly affect the results. Detailed results are shown in the tables below.

**Table 3**  
Outcomes at Month 12 and Month 24 in Study 1

Outcome Measure	Month	Sham n = 238	LUCENTIS 0.5 mg n = 240	Estimated Difference (95% CI) <sup>a</sup>
Loss of < 15 letters in visual acuity (%) <sup>b</sup>	Month 12	62%	95%	32% (26%, 39%)
	Month 24	53%	90%	37% (29%, 44%)
Gain of ≥ 15 letters in visual acuity (%) <sup>b</sup>	Month 12	5%	34%	29% (22%, 35%)
	Month 24	4%	33%	29% (23%, 35%)
Mean change in visual acuity (letters) (SD) <sup>b</sup>	Month 12	-10.5 (16.6)	+7.2 (14.4)	17.5 (14.8, 20.2)
	Month 24	-14.9 (18.7)	+6.6 (16.5)	21.1 (18.1, 24.2)

<sup>a</sup> Adjusted estimate based on the stratified model.

<sup>b</sup> p < 0.01.

**Table 4**  
Outcomes at Month 12 in Study 2

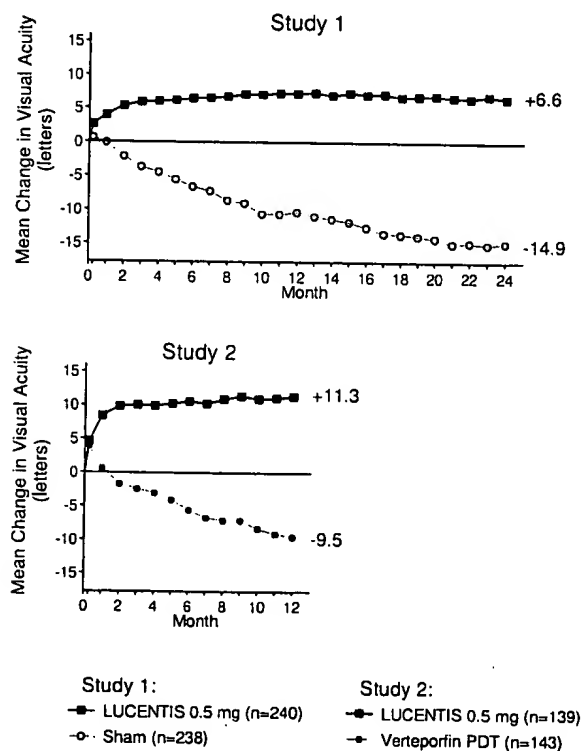
Outcome Measure	Verteporfin PDT n = 143	LUCENTIS 0.5 mg n = 140	Estimated Difference (95% CI) <sup>a</sup>
Loss of < 15 letters in visual acuity (%) <sup>b</sup>	64%	96%	33% (25%, 41%)
Gain of ≥ 15 letters in visual acuity (%) <sup>b</sup>	6%	40%	35% (26%, 44%)
Mean change in visual acuity (letters) (SD) <sup>b</sup>	-9.5 (16.4)	+11.3 (14.6)	21.1 (17.5, 24.6)

<sup>a</sup> Adjusted estimate based on the stratified model.

<sup>b</sup> p < 0.01.

**Figure 1**

Mean Change in Visual Acuity from Baseline to Month 24 in Study 1 and to Month 12 in Study 2



Patients in the group treated with LUCENTIS had minimal observable CNV lesion growth, on average. At Month 12, the mean change in the total area of the CNV lesion was 0.1-0.3 DA for LUCENTIS versus 2.3-2.6 DA for the control arms.

The use of LUCENTIS beyond 24 months has not been studied.

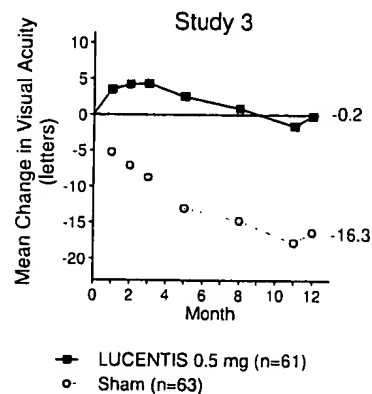
#### 14.2 Study 3

Study 3 was a randomized, double-masked, sham-controlled, two-year study designed to assess the safety and efficacy of LUCENTIS in patients with neovascular AMD (with or without a classic CNV component). Data are available through Month 12. Patients received LUCENTIS 0.3 mg or 0.5 mg intravitreal injections or sham injections once a month for 3 consecutive doses, followed by a dose administered once every 3 months. A total of 184 patients were enrolled in this study (LUCENTIS 0.3 mg, 60; LUCENTIS 0.5 mg, 61; sham, 63); 171 (93%) completed 12 months of this study. Patients treated with LUCENTIS in Study 3 received a mean of 6 total treatments out of possible 6 from Day 0 through Month 12.

In Study 3, the primary efficacy endpoint was mean change in visual acuity at 12 months compared with baseline (see Figure 2). After an initial increase in visual acuity (following monthly dosing), on average, patients dosed once every three months with LUCENTIS lost visual acuity, returning to baseline at Month 12. In Study 3, almost all LUCENTIS-treated patients (90%) maintained their visual acuity at Month 12.

**Figure 2**

Mean Change in Visual Acuity from Baseline to Month 12 in Study 3



#### 16 HOW SUPPLIED/STORAGE AND HANDLING

Each LUCENTIS carton, NDC 50242-080-01, contains one 2-cc glass vial of ranibizumab, one 5-micron, 19-gauge × 1-1/2-inch filter needle for withdrawal of the vial contents, one 30-gauge × 1/2-inch injection needle for the intravitreal injection, and one package insert [see Dosage and

*Administration (2.4)]. VIALS ARE FOR SINGLE EYE USE ONLY.*

**17 PATIENT COUNSELING INFORMATION**

In the days following LUCENTIS administration, patients are at risk of developing endophthalmitis. If the eye becomes red, sensitive to light, painful, or develops a change in vision, the patient should seek immediate care from an ophthalmologist [see *Warnings and Precautions (5.1)*].

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**LUCENTIS™ [ranibizumab injection]**

Manufactured by: 8277700

Genentech, Inc. LL1404

1 DNA Way 4833801

South San Francisco, CA 94080-4990 FDA Approval Date:

June 2006

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Inc.





DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration  
Rockville, MD 20852

BLA 125156

Genentech, Inc.  
Attention: Robert L. Garnick, Ph.D.  
Senior Vice President, Regulatory Affairs, Quality & Compliance  
1 DNA Way  
South San Francisco, California 94080-4990

Dear Dr. Garnick:

We have approved your biologics' license application for Lucentis (ranibizumab injection) effective this date. You are hereby authorized to introduce or deliver for introduction into interstate commerce, ranibizumab injection under your existing Department of Health and Human Services U.S. License No. 1048. Lucentis (ranibizumab injection) is indicated for the treatment of patients with neovascular (wet) age-related macular degeneration.

Under this license, you are approved to manufacture ranibizumab drug substance at Genentech, Inc., South San Francisco, California; fill the final formulated product at (b) (4) and label and package filled vials at Genentech, Inc., South San Francisco, California. You may label your product with the proprietary name Lucentis and market it in 10 mg/mL single use glass vials.

We acknowledge receipt of your submissions dated December 29, 2005, and January 31, February 10, 17, 21, and 24, March 17, 23, and 31, April 10, and 28, May 5, 10, 25 (2), 26 (2), and 31, and June 1, 5 (2), 6, 9, 13, 16, 23, 26, 27, 28 (3), and 29, 2006.

The final printed labeling (FPL) must be identical in content to the enclosed labeling text for the package insert, submitted June 28, 2006; the immediate vial container submitted March 31, 2006; and the carton labels submitted June 5, 2006. The statement "No U.S. standard of potency" should be added with the next printing of carton labels. Marketing this product with FPL that is not identical in content to the approved labeling text may render the product misbranded and an unapproved new drug.

The dating period for formulated drug product shall be 18 months from the date of manufacture when stored at 2°-8°C (36°-46°F). The date of manufacture shall be defined as the date of final sterile filtration of the formulated drug product. The dating period for ranibizumab drug substance shall be (b) (4) when stored at -20 °C.

You currently are not required to submit samples of future lots of Lucentis to the Center for Drug Evaluation and Research (CDER) for release by the Director, CDER, under 21 CFR 610.2. We will continue to monitor compliance with 21 CFR 610.1 requiring completion of tests for conformity with standards applicable to each product prior to release of each lot.

You must submit information to your biologics license application for our review and written approval under 21 CFR 601.12 for any changes in the manufacturing, testing, packaging or labeling of Lucentis, or in the manufacturing facilities.

All applications for new active ingredients, new dosage forms, new indications, new routes of administration, and new dosing regimens are required to contain an assessment of the safety and effectiveness of the product in pediatric patients unless this requirement is waived or deferred. We are waiving the pediatric study requirement for this application.

The following are Postmarketing Studies that are subject to reporting requirements of 21 CFR 601.70:

1. Submit the final Clinical Study Report from Study FVF3689g by June 30, 2008.
2. Provide safety and efficacy data from a 2-year adequate and well-controlled clinical trial of a mutually acceptable design exploring multiple dosing frequencies of Lucentis.

Date of submission of protocol: November 14, 2008.

Date of start of study: September 21, 2009.

Date of final clinical study report: April 1, 2013.

3. To detect and characterize immune responses to ranibizumab:
  - a. Develop and validate a confirmatory assay capable of detecting both IgG and IgM isotype responses.
  - b. Develop and validate an assay to detect neutralizing anti-ranibizumab antibodies.

The assay methodology and validation reports: September 28, 2007.

4. To characterize further the immune response to ranibizumab, serum samples collected in studies FVF2587g, FVF2598g, FVF3192g will be assayed using the validated methods described above in Postmarketing Commitment #3. The data obtained will be analyzed to discover and evaluate any association between immunoreactivity and dosing frequency as well as any potential impact of immunoreactivity on efficacy or safety outcomes.

The need for an additional clinical study will be determined based on the results from the analysis described above.

Date of submission of protocol and statistical analysis plan: February 28, 2007.

Date of submission of final study report: September 30, 2008.

The following are Postmarketing Studies that are not subject to reporting requirements of 21 CFR 601.70:

5. To revise release specifications, shelf-life specifications and in-process limits for ranibizumab drug substance and drug product after (b) (4) commercial manufacturing runs to reflect increased manufacturing experience.

These revisions to the Quality control system, the corresponding data from the (b) (4) commercial manufacturing runs and the analysis plan used to create the revisions will be submitted as a supplement on or before June 30, 2008.

6. To perform additional Lucentis stability studies at 40°C using Ion Exchange Chromatography (IEC) to demonstrate that the corrective actions taken at (b) (4) to address the atypical accelerated stability profile observed in the Lucentis 2005 qualification campaign have been sufficient.

Specifically, a one time stability study consisting of (b) (4) centis Drug Product launch lots are placed at 40°C and tested by IEC at (b) (4) months. These (b) (4) Lucentis Drug Product lots are derived from the following:

- (b) (4) of these Lucentis Drug Product lots are manufactured from distinct lots of (b) (4).
- At least (b) (4) these (b) (4) lots are aliquoted and used to manufacture (b) (4) Lucentis drug product lots.

Data will be submitted as a supplement on or before March 31, 2007.

We request that you submit clinical protocols to your IND, with a cross-reference letter to this biologics license application. Submit nonclinical and chemistry, manufacturing, and controls protocols and all study final reports to this application. Please use the following designators to label prominently all submissions, including supplements, relating to these postmarketing study commitments as appropriate:

- **Postmarketing Study Protocol**
- **Postmarketing Study Final Report**
- **Postmarketing Study Correspondence**
- **Annual Report on Postmarketing Studies**

For each postmarketing study subject to the reporting requirements of 21 CFR 601.70, you must describe the status in an annual report on postmarketing studies for this product. The status report for each study should include:

- information to identify and describe the postmarketing commitment,
- the original schedule for the commitment,
- the status of the commitment (i.e. pending, ongoing, delayed, terminated, or submitted),

- an explanation of the status including, for clinical studies, the patient accrual rate (i.e. number enrolled to date and the total planned enrollment), and
- a revised schedule if the study schedule has changed and an explanation of the basis for the revision.

As described in 21 CFR 601.70(e), we may publicly disclose information regarding these postmarketing studies on our Web site (<http://www.fda.gov/cder/pmc/default.htm>). Please refer to the April 2001 Draft Guidance for Industry: Reports on the Status of Postmarketing Studies – Implementation of Section 130 of the Food and Drug Administration Modernization Act of 1997 (see <http://www.fda.gov/cber/gdlns/post040401.htm>) for further information.

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If you have any questions, call Lori M. Gorski, Project Manager, at (301) 796-0722.

Sincerely,

Mark J. Goldberger, M.D., M.P.H.  
Director  
Office of Antimicrobial Products  
Center for Drug Evaluation and Research

Enclosure



UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 6,884,879 B1  
DATED : April 26, 2005  
INVENTOR(S) : Baca et al.

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

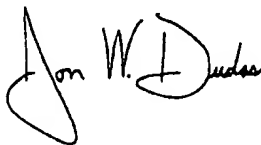
Title page.

Item [75], Inventors, should read as follows:

-- Manuel Baca, Foster City, CA (US);  
James A. Wells, Burlingame, CA (US);  
Leonard G. Presta, San Francisco, CA (US);  
Henry B. Lowman, El Granada, CA (US);  
Yvonne Man-yea Chen, San Mateo, CA (US) --.

Signed and Sealed this

Twenty-third Day of August, 2005



---

ION W. DUDAS  
*Director of the United States Patent and Trademark Office*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT

In re Patent Application of:

Manuel Baca, et al.

Serial No.: 08/908,469

Filed: April 6, 1997

For: ANTI-VEGF ANTIBODIES



Examiner Susan Ungar  
Group Unit 1642

*[Handwritten signatures and initials]*

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on January 28, 1999.

Signed:

*[Signature of Judy Wilson]*  
Judy Wilson

Date: January 28, 1999

AMENDMENT

Assistant Commissioner for Patents  
Washington, DC 20231

Sir:

This is in response to the Official Action mailed August 31, 1998. Applicants respectfully request a two month extension of time. The Commissioner is authorized to charge any additional fees, including extensions of time, which may be required, or any overpayment to Deposit Account No. 06-1300, (Order No. A-65358/WHD/DAV).

Please amend the application as follows and consider the remarks set forth thereafter.

In the specification:

On page 1, line 10, after "1997," please insert, "converted to a provisional application April 6, 1998,".

02/11/1999 BFRISON 00000002 061300 08908469  
01 FC:102 .78.00 CH





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08/833,504 HUMANIZED ANTIBODIES AND METHODS FOR FORMING HUMANIZED ANTIBODIES

Application Transaction History Continuity Data Address & Attorney/Agent

## Bibliographic Data

Application Number:	08/833,504	Customer Number:	-
Filing or 371 (c) Date:	04-07-1997	Status:	Utility Application Converted Provisional Application
Application Type:	Utility	Status Date:	09-20-2001
Examiner Name:	UNGAR, SUSAN NMN	Location:	OIPE - INITIAL EXAM MANAGER'S OFFICE, ST - 7A CORRIDOR
Group Art Unit:	1642	Location Date:	05-17-1999
Confirmation Number:	2613	Earliest Publication No:	-
Attorney Docket Number:	A-64254/WHD/	Earliest Publication Date:	-
Class / Subclass:	424/130.100	Patent Number:	-
First Named Inventor:	James A. Wells , Burlingame, CA (US)	Issue Date of Patent:	-

Title of Invention: HUMANIZED ANTIBODIES AND METHODS FOR FORMING HUMANIZED ANTIBODIES

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08/833,504 HUMANIZED ANTIBODIES AND METHODS FOR FORMING HUMANIZED ANTIBODIES

Application Data	Transaction History	Continuity Data	Address & Attorney/Agent	Supplemental Content
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## Transaction History

Date	Transaction Description
04-10-1999	Workflow - File Sent to Contractor
09-20-2001	Set Application Status
05-17-1999	Pre_Exam Conversion
04-23-1999	Sequence Forwarded to Pubs on Tape
04-21-1999	Workflow - Query Request - Begin
06-05-1998	Mail Express Abandonment (During Examination)
05-29-1998	Express Abandonment (during Examination)
04-08-1998	Petition Entered
03-23-1998	Information Disclosure Statement (IDS) Filed
04-14-1998	Case Docketed to Examiner in GAU
04-06-1998	Date Forwarded to Examiner
03-18-1998	Response after Non-Final Action
03-18-1998	Request for Extension of Time - Granted
01-22-1998	Information Disclosure Statement (IDS) Filed
11-18-1997	Mail Non-Final Rejection
11-05-1997	Non-Final Rejection

09-11-1997	Case Docketed to Examiner in GAU
09-04-1997	Application Dispatched from OIPE
09-02-1997	Application Is Now Complete
06-03-1997	Notice Mailed--Application Incomplete--Filing Date Assigned
06-02-1997	CRF Is Good Technically / Entered into Database
05-27-1997	IFW Scan & PACR Auto Security Review
05-21-1997	CRF Disk Has Been Received by Preexam / Group / PCT
05-08-1997	Initial Exam Team nn

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RJ

Patent Docket P1093P1

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of  Baca, Manuel, et al.  U.S. Patent No.: 6,884,879  Issued: April 26, 2005  For: Anti-Vegf Antibodies	Confirmation No: 9546  CUSTOMER NO: 09157
	<p><b>CERTIFICATE OF MAILING</b></p> <p>I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on</p> <p>May 19, 2006</p> <p><i>Barbara Hebert</i> Barbara Hebert</p>

REQUEST FOR CERTIFICATE OF CORRECTION UNDER 37 CFR 1.322

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

Enclosed is a Certificate of Correction for the above-referenced patent. Because the mistake occurred in the printing of the patent, it is not believed that any fee is required. However, if this is not the case, the Commissioner is hereby authorized to charge the required fee to Deposit Account No. 07-0630. Acceptance of this Certificate of Correction is respectfully requested.

Respectfully submitted,  
GENENTECH, INC.

Date: May 19, 2006

By: *Steven X. Cui*  
Steven X. Cui  
Reg. No. 44,637  
Telephone No. (650) 225-8674

Certificate  
of Correction  
MAY 25 2006

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 6,884,879 B1

DATED : April 26, 2004

INVENTOR(S) : Manuel Baca et al.

It is certified that an error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title Page

Item(63), Related U.S. Application Data should read as follows:

The present application claims benefit of provisional patent application No. 60/126,446, filed April 7, 1997, entitled: "Humanized Antibodies and Methods for Forming Humanized Antibodies", by inventors Manuel Baca et al., the disclosure of which is hereby incorporated by reference.

MAILING ADDRESS OF SENDER:

Steven X. Cui  
Genentech, Inc.  
1 DNA Way  
So. San Francisco, CA 94080-4990

PATENT NO. 6,884,879

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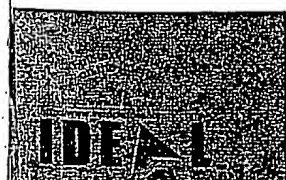
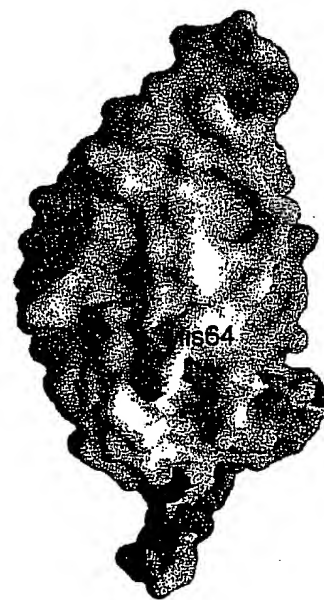
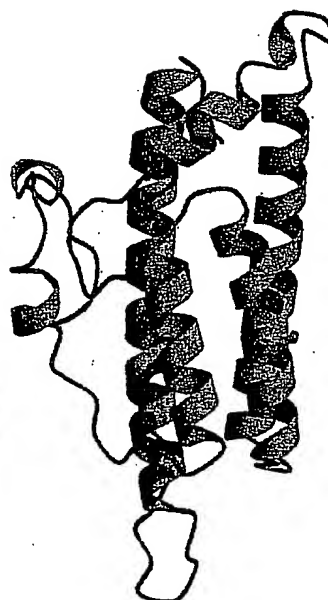


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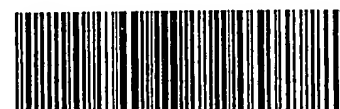
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## Selection and Analysis of an Optimized Anti-VEGF Antibody: Crystal Structure of an Affinity-matured Fab in Complex with Antigen

Yvonne Chen<sup>1</sup>, Christian Wiesmann<sup>1</sup>, Germaine Fuh<sup>1</sup>, Bing Li<sup>1</sup>,  
Hans W. Christinger<sup>1</sup>, Patrick McKay<sup>2</sup>, Abraham M. de Vos<sup>1</sup>  
and Henry B. Lowman<sup>1\*</sup>

<sup>1</sup>Department of Protein Engineering, Genentech, Inc.  
1 DNA Way, South San Francisco, CA 94080, USA

<sup>2</sup>Department of Process Sciences, Genentech, Inc.  
1 DNA Way, South San Francisco, CA 94080, USA

The Fab portion of a humanized antibody (Fab-12; IgG form known as rhuMAB VEGF) to vascular endothelial growth factor (VEGF) has been affinity-matured through complementarity-determining region (CDR) mutation, followed by affinity selection using monovalent phage display. After stringent binding selections at 37°C, with dissociation (off-rate) selection periods of several days, high affinity variants were isolated from CDR-H1, H2, and H3 libraries. Mutations were combined to obtain cumulatively tighter-binding variants. The final variant identified here, Y0317, contained six mutations from the parental antibody. *In vitro* cell-based assays show that four mutations yielded an improvement of about 100-fold in potency for inhibition of VEGF-dependent cell proliferation by this variant, consistent with the equilibrium binding constant determined from kinetics experiments at 37°C. Using X-ray crystallography, we determined a high-resolution structure of the complex between VEGF and the affinity-matured Fab fragment. The overall features of the binding interface seen previously with wild-type are preserved, and many contact residues are maintained in precise alignment in the superimposed structures. However, locally, we see evidence for improved contacts between antibody and antigen, and two mutations result in increased van der Waals contact and improved hydrogen bonding. Site-directed mutants confirm that the most favorable improvements as judged by examination of the complex structure, in fact, have the greatest impact on free energy of binding. In general, the final antibody has improved affinity for several VEGF variants as compared with the parental antibody; however, some contact residues on VEGF differ in their contribution to the energetics of Fab binding. The results show that small changes even in a large protein-protein binding interface can have significant effects on the energetics of interaction.

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\*Corresponding author

**Keywords:** angiogenesis; humanized antibody-antigen complex; affinity maturation; phage display; X-ray crystallography

Abbreviations used: CDR, complementarity-determining region; FR, framework region; HuVEC, human umbilical vein endothelial cell;  $K_d^{25}$ , equilibrium dissociation constant determined at 25°C; mAb, IgG form of monoclonal antibody; PBS, phosphate-buffered saline; SPR, surface plasmon resonance; VEGF, vascular endothelial growth factor; VEGF(109), receptor-binding fragment of VEGF with residues 8-109; VEGF(165), VEGF form with residues 1-165.

E-mail address of the corresponding author:  
hbl@gene.com

### Introduction

Angiogenic factors (Folkman & Klagsbrun, 1987), which stimulate endothelial cells leading to new vascularization, have roles in such disease states as cancer, rheumatoid arthritis, and macular degeneration (reviewed by Ferrara, 1995; Folkman, 1995; Iruela-Arispe & Dvorak, 1997). Vascular endothelial growth factor (VEGF), a heparin-binding protein initially identified from pituitary cells (Ferrara & Henzel, 1989), is clearly a key angio-

genic factor in development as well as in certain disease states, including the growth of solid tumors (reviewed by Ferrara, 1999). A murine monoclonal antibody, A.4.6.1, was found to block VEGF-dependent cell proliferation *in vitro* and to antagonize tumor growth *in vivo* (Kim *et al.*, 1993). The murine mAb was previously humanized in Fab form to yield a variant known as Fab-12 (Presta *et al.*, 1997). Both chimeric and humanized antibodies retained high affinity binding to VEGF, with an apparent equilibrium dissociation constant,  $K_d^{25^\circ}$ , of 0.9 to 3 nM (Presta *et al.*, 1997; Baca *et al.*, 1997; Muller *et al.*, 1998a). The corresponding full-length IgG form of this antibody, rhumAb VEGF, is being developed as a possible therapeutic agent for the treatment of human solid tumors (Mordenti *et al.*, 1999).

We became interested in obtaining higher affinity variants of Fab-12 in order to test whether affinity improvements of this antibody might improve its potency and efficacy. Phage display of randomized libraries of antibodies and other proteins has been extensively used to engineer proteins with improved affinity and specificity (Lowman *et al.*, 1991; reviewed by Kay & Hoess, 1996; Rader & Barbas, 1997; Griffiths & Duncan, 1998). In particular, a phage-based *in vitro* affinity maturation process has been successful in improving the binding affinity of antibodies previously identified from traditional monoclonal or naive-library sources (e.g. Hawkins *et al.*, 1992; Marks *et al.*, 1992; Barbas *et al.*, 1994; Yang *et al.*, 1995; Schier *et al.*, 1996; Thompson *et al.*, 1996).

In previous work, the humanized anti-VEGF antibody Fab-12 was adapted for improved monovalent phage display through selection of a CDR-L1 variant, designated Y0192 (Muller *et al.*, 1998a). To select target residues for randomization and affinity optimization, we also previously screened all CDR residues, as defined by a combination of the hypervariable (Kabat *et al.*, 1987) and structurally defined (Chothia & Lesk, 1987) CDR residues. Fab variants of Y0192 generated by alanine scanning were analyzed for side-chain contributions to antigen binding (Muller *et al.*, 1998a). In addition, a crystal structure of Fab-12 in complex with the receptor-binding domain of VEGF, VEGF(109), was determined (Muller *et al.*, 1998a). The results of these studies showed that the antigen binding site is almost entirely composed of residues from the heavy chain CDRs, CDR-H1, H2, and H3. Therefore, these CDRs appeared most likely to provide the opportunity for improved binding interactions with antigen.

Here, we describe the selection of an affinity-improved anti-VEGF antibody by phage display and off-rate selection. We show that the affinity-matured antibody binds VEGF with at least 20-fold improved affinity and inhibits VEGF-induced cell proliferation with enhanced potency in a cell-based assay. We also report the crystal structure of an affinity-optimized antibody in complex with VEGF, to our knowledge, representing the first

reported structure of an *in vitro* affinity-matured antibody:antigen complex. The structure, together with mutational analysis, shows that subtle changes in the antibody-antigen interface account for improved affinity.

## Results

### Library design

We used the results of an alanine-scanning analysis, combined with a crystal structure of the wild-type Fab fragment in complex with VEGF (Muller *et al.*, 1998a), to design targeted libraries within the antibody CDRs for random mutagenesis and affinity selection. This strategy enabled us to construct theoretically complete libraries with a small number of residues randomized in each CDR. Although sites remote from the antigen-combining region or buried within the protein could modulate antigen binding affinity indirectly and have in fact been exploited for affinity improvement (Hawkins *et al.*, 1993), clearly residues shown to be important by alanine scanning are useful starting points for binding-affinity optimization (Lowman *et al.*, 1991; Lowman & Wells, 1993). Furthermore, we reasoned that by making mutations at residues of the antibody CDRs which were known to affect antigen binding and were located at or near points of contact in the bound complex, we could minimize the possibility of other indirect effects which might alter stability, immunogenicity, or other properties of the antibody.

Both Ala-scanning and crystallography (Muller *et al.*, 1998a) identified CDR-H3 as the predominant contact segment for VEGF, consistent with the general observation that CDR-H3 is often key to antigen binding (Chothia & Lesk, 1987). Within CDR-H3, residues Y95, P96, H97, Y98, Y99, S100b, H100c, W100d, Y100e, and F100f (numbering is as described by Kabat *et al.* (1987)), all showed effects on binding over a range of twofold to >150-fold when mutated to Ala, and Ala substitution at S100a caused a slight improvement in binding. However, H100c, Y100e, and F100f were found to have little or no direct contact with VEGF and presumed to have indirect effects on binding. On the other hand, Y95 and W100d have significant contacts with VEGF, and Ala substitutions resulted in no detectable binding to VEGF. Therefore, these residues were excluded from optimization. Inspection of the complex structure suggested that substitutions at P96 and Y98 could be disruptive to the antibody structure, while G100, where Ala mutation had little effect, might tolerate further substitutions. We therefore constructed a library (YC81) which fully randomized positions H97, Y99, G100, S100a, and S100b, within CDR-H3.

Significant effects of Ala substitution were also found in CDR-H2. Here, W50, I51, N52, T52a, Y53, T54, T58 alanine mutants all showed >twofold loss in binding affinity, with the greatest residue surface area buried at positions W50, I51, Y53, and



T58 (Muller *et al.*, 1998a). Indeed, W50 along with other aromatic side-chains was observed to form a deep pocket into which a loop of VEGF inserts in the complex, and was excluded from further optimization. Residue I51, on the other hand, showed no direct contact with VEGF and was also excluded. Residue T58 had multiple interactions within the interface, including contacts with VEGF and with the critical W50 of the CDR pocket. Although E56 showed no contact with VEGF and little effect (<twofold) upon alanine substitution, its side-chain lies at the periphery of the interface, near several hydrophobic residues of VEGF. We reasoned that these might be exploited for additional binding interactions. Two CDR-H2 libraries were constructed: YC266, randomizing positions T52a, Y53, T54, and E56; and YC103, randomizing positions N52, T52a, Y53, and T54.

In CDR-H1 G26, Y27, F29, N31, Y32, G33, M34, and N35 were implicated by alanine mutagenesis as important for binding VEGF; however, only N31, Y32, and G33 had significant direct contacts with VEGF. Since Ala substitution of G33 showed reduced binding, larger side-chains seemed less desirable; for this reason, this position was not randomized. Residues 27 (buried in the antibody structure) and T28 and T30 (which are mutually contacting) were included at the end of the H1 loop as possible indirect determinants of binding. Residues 27, 28, and 30-32 were randomized in a library designated YC265.

Framework residues, especially heavy chain residues 71 and 93, normally outside the region of contact with antigen, have also been found to affect antibody binding affinity (Tramontano *et al.*, 1990; Foote & Winter, 1992; Hawkins *et al.*, 1993; Xiang *et al.*, 1995), and sometimes participate in antigen contacts (reviewed by Nezhlin, 1998). Therefore, an additional region of the anti-VEGF Fab, within FR-H3 and including position 71, was also targeted for randomization. Since the residue 71-76 region has contacts with CDR-H1 (at F29) and CDR-H2 (at I51 and T52a), these represented potential sites for affi-

nity improvement through secondary effects on the interface residues. Residues L71, T73, and S76 were randomized in this FR-H3 library.

### Phage selections

Fab libraries were constructed using a fusion to the g3p minor coat protein in a monovalent phage display (phagemid) vector (Bass *et al.*, 1990; Lowman *et al.*, 1991). For each library, stop codons were introduced by mutagenesis into the Y0192 phage template (Muller *et al.*, 1998a) at each residue position to be randomized. Each stop-codon construct was then used for construction of a fully randomized (using NNS codons) library as described in Materials and Methods. Phage were precipitated from overnight *Escherichia coli* shake-flask cultures and applied to VEGF-coated immunosorbant plates for binding selections. Cycles of selection followed by amplification were carried out essentially as described (Lowman, 1998).

We used an off-rate selection process (see Materials and Methods) similar to previously described procedures (Hawkins *et al.*, 1992; Yang *et al.*, 1995), modified by gradually increasing the selective pressure for binding to antigen during successive cycles of enrichment. The enrichment factor (ratio of displaying phage to non-displaying phage eluted *versus* applied) was used to monitor the stringency of selection at each step (Table 1). As a control, and to obtain a relative measure of affinity improvement, Y0192-phage were subjected to the same procedure at each cycle.

Fab-phage clones were sequenced from several phage-binding selection rounds that showed enrichment for Fab-phage over non-displaying phage. From round 6 of the CDR-H1 library selections, a dominant clone, Y0243-1 was found, having wild-type residues at Y27, T30, and Y32, and substitutions T28D and N31H (Table 2). Additional clones had related sequences, with N31H found in all selectants; Asp or Glu substituting for T28; and Thr, Ser, Gln, or Gly found at position T30.

Table 1. Enrichment factors from phage-displayed Fab libraries

Round	Wash time (hours)	CDR-H1 YC265	CDR-H2 YC266	CDR-H2 YC103	CDR-H3 YC81	FR-H3 YC101	Control Y0192
1	0	8.2	1.7	1.3	3.3	4	1.5
2	1	1.6	25	0.7	10	110	90
3	2	340	880	100	570	2300	22000
4	18	6800	880	5200	3700	600	2700
5	37 <sup>a</sup>	210	900	920	1300	480	32
6	47 <sup>a</sup>	130	80	100	3500	30	20
7	63 <sup>a</sup>	1	1	>3	>25	1	>8

Libraries are designated by CDR region and oligonucleotide label (see the text for details). Library Fab-phage (ampicillin-resistant) were mixed with non-displaying control phage (chloramphenicol-resistant) in each starting pool, and subjected to VEGF binding selection, washing, and elution as described in the text.

The enrichment factor for each library is reported here as the ratio of Amp/Cam colony-forming units in the eluted pool, divided by the ratio of Amp/Cam colony-forming units in the starting pool. Starting phage concentrations were about  $10^{12}$ /ml, except  $10^{13}$ /ml in round 1. The wild-type Fab-phage, Y0192 was included at each round for comparison of enrichment under the particular conditions used.

<sup>a</sup> In some cases, the wash-step included incubation at 37°C.

Table 2. Anti-VEGF Fab variants selected from a CDR-H1 library (HL-265)

Variant	n	Y 27	T 28	T 30	N 31	Y 32	I 34 <sup>a</sup>	$K_d(Y0192)/K_d(\text{variant})$
Round 6 (HCl)								
Y0243-1	5	Y	D	T	H	Y	M	3.1
Y0243-2	1	Y	E	Q	H	Y	M	
Y0243-3	1	Y	E	T	H	Y	M	
Y0243-4	1	Y	D	G	H	Y	M	
Y0243-5	1	Y	D	S	H	Y	M	
Y0243-6	1	Y	E	S	H	Y	M	
Consensus:		Y	D	T	H	Y	M	3.1

All variants are in the background of Y0192 (Muller *et al.*, 1998a). *n* indicates the number of clones found with identical DNA sequence. The wild-type (Y0192) residue is shown at the top of each column, and the sequence position number is indicated according to Kabat *et al.* (1987).

<sup>a</sup> Position 34 was not randomized, but was changed to Met (as in Fab-12) in this library. The consensus reported here, equivalent to clone Y0243-1, represents the most abundant amino acid residue at each position (including clones with multiple representation (*n* > 1)).  $K_d(Y0192)/K_d(\text{variant})$  indicates the fold increase in binding affinity *versus* the wild-type humanized antibody Y0192 (see Table 6).

Clones from two independently constructed CDR-H2 libraries were remarkable in that all sequenced library clones conserved wild-type residues at virtually all positions mutated, except at position Y53, where all clones contained a Trp substitution (Table 3).

Because of the strong enrichment observed from the CDR-H3 library, a number of clones were sequenced from rounds 5 and 7 (Table 4). Of 39 sequenced clones, 37 retained the wild-type residue S100b, and all contained the mutation H97Y. The remaining positions showed greater diversity, even after seven cycles of selection. The dominant clone at round 7, Y0238-3, contained the mutation S100aT (in addition to H97Y), with wild-type residues Y99 and G100. Other substitutions observed included Lys or Arg for Y99 (in 18 of 39 clones), G100N (11 of 39 clones), and a variety of substitutions including Arg, Glu, Gln, and Asn at S100a. In this library, the consensus sequence is represented by the dominant clone, Y0238-1 (Table 4).

Clones from round 6 of the FR-H3 library (Table 5) showed conservation of wild-type residue S76, with wild-type residues or various substi-

tutions at the remaining positions: Val or Ile substituting for L71, and Val or Lys substitutions at T73.

### Binding affinity of selected variants

For measurements of binding affinity, we made use of an amber stop codon placed between the genes for the Fab heavy chain and the g3p C-terminal domain, and expressed soluble Fab variants from *E. coli* shake-flask or fermentation cultures. Fab variants purified from protein-G affinity chromatography were characterized for binding affinity using an SPR-based assay on a BIAcore™-2000 instrument. The binding-kinetics assay has been described (Muller *et al.*, 1998a).

Association kinetics ( $k_{on}$ ) for the wild-type antibody binding to immobilized VEGF are slow (Presta *et al.*, 1997; Baca *et al.*, 1997; Muller *et al.*, 1998a), and none of the variants tested had significantly improved on-rates. On the other hand, dissociation kinetics varied over a range of  $10^{-4}$  s<sup>-1</sup> to  $\leq 4 \times 10^{-6}$  s<sup>-1</sup> at 25°C (Table 6). Based on measurements of instrumental drift, we could not accurately measure  $k_{off}$  (and consequently  $K_d$ )

Table 3. Anti-VEGF Fab variants selected from CDR-H2 libraries (HL-266, YC103)

Variant	n	N 52 <sup>a</sup>	T 52a	Y 53	T 54	G 55 <sup>a,b</sup>	E 56 <sup>a</sup>	$K_d(Y0192)/K_d(\text{variant})$
Round 6 (HCl)								
HL266-A <sup>b</sup>	6	N	T	W	T	G	E	1.3
HL266-E	1	N	T	W	T	G	T	
HL266-I	1	N	T	W	T	G	Q	
YC103-A <sup>b</sup>	7	N	T	W	T	G	E	1.3
YC103-C	1	N	T	W	D	G	E	
Consensus		N	T	W	T	G	E	1.3

All variants are in the background of Y0192 (Muller *et al.*, 1998a). *n* indicates the number of clones found with identical DNA sequence. The wild-type (Y0192) residue is shown at the top of each column, and the sequence position number is indicated according to Kabat *et al.* (1987). The consensus reported here, equivalent to clones HL266A and YC103A, represents the most abundant amino acid at each position (including clones with multiple representation; i.e. *n* > 1).  $K_d(Y0192)/K_d(\text{variant})$  indicates the fold increase in binding affinity *versus* the wild-type humanized antibody Y0192 (see Table 6).

<sup>a</sup> Constant positions were position 52 in the HL-266 library and position 56 in the YC103 library.

<sup>b</sup> Equivalent clones are assumed to have equal affinity.

Table 4. Anti-VEGF Fab variants selected from a CDR-H3 library (YC81)

Variant	n	H 97	Y 99	G 100	S 100a	S 100b	$K_d(\text{Y0192})/K_d(\text{variant})$
Round 5 (VEGF)							
Y0228-21	1	Y	R	N	A	S	
Y0228-22	1	Y	T	T	R	S	
Y0228-23	1	Y	E	G	S	S	
Y0228-24	1	Y	R	Q	R	G	
Y0228-26	1	Y	T	G	R	S	
Y0228-27	1	Y	T	N	T	S	
Y0228-28	1	Y	R	K	G	S	
Y0228-29	1	Y	T	G	S	S	
Y0228-30	1	Y	R	S	G	S	
Round 5 (HCl)							
Y0229-20	1	Y	T	N	R	S	
Y0229-21	1	Y	R	N	S	S	
Y0229-22	1	Y	K	E	S	S	
Y0229-23	1	Y	R	D	A	S	
Y0229-24	1	Y	R	Q	K	G	
Y0229-25	1	Y	K	G	G	S	
Y0229-26	1	Y	Y	G	A	S	
Y0229-27	1	Y	R	G	E	S	
Y0229-28	1	Y	R	S	T	S	
Y0238-10 <sup>a</sup>	1	Y	R	N	T	S	3.8
Round 7 (HCl)							
Y0238-3	6	Y	Y	G	T	S	≥9.4
Y0238-1	2	Y	R	G	T	S	7.3
Y0238-2	2	Y	I	N	K	S	
Y0238-10 <sup>a</sup>	2	Y	R	N	T	S	3.8
Y0238-4	1	Y	Y	N	Q	S	
Y0238-5	1	Y	I	A	K	S	2.1
Y0238-6	1	Y	R	D	N	S	≥5.4
Y0238-7	1	Y	W	G	T	S	
Y0238-8	1	Y	R	Q	N	S	
Y0238-9	1	Y	R	Q	S	S	
Y0238-11	1	Y	K	N	T	S	
Y0238-12	1	Y	I	E	R	S	
Consensus		Y	R	G	T	S	7.3

All variants are in the background of Y0192 (Müller *et al.*, 1998a). The clones are grouped according to the round of selection (5 or 7) and the type of elution (VEGF competition or HCl elution) used for recovery of bound phage. *n*, indicates the number of clones found with identical DNA sequence within each group. The wild-type (Fab-12, or Y0192) residue is shown at the top of each column, and the sequence position number is indicated according to Kabat *et al.* (1987). The consensus reported here, equivalent to clone Y0238-1, represents the most abundant amino acid at each position (including clones with multiple representation (*n* > 1)).  $K_d(\text{Y0192})/K_d(\text{variant})$  indicates the fold increase in binding affinity *versus* the wild-type humanized antibody Y0192 (see Table 6).

<sup>a</sup> One clone was identified at both rounds 5 and 7. Equivalent clones are assumed to have equal affinity.

under these conditions, but instead used the kinetics data to place an upper limit on  $K_d$ .

The phage-derived Fab variants tested showed a range of small (within experimental error of about twofold) to significant (>fivefold) improvements in binding affinity over the wild-type (parental phage) antibody Y0192 (Table 6). From the CDR-

H1 library, the dominant clone (Y0243-1) showed threefold improved affinity. Variant Y0242-1, the dominant clone in each of three CDR-H2 libraries, showed an affinity equivalent to wild-type within experimental error, and two clones derived from the FR-H3 library (Y0244-1 and Y0244-4) were equivalent or slightly weaker in affinity. Small

Table 5. Anti-VEGF Fab variants selected from a FR-H3 library

Variant	n	L 71	T 73	S 76	$K_d(\text{Y0192})/K_d(\text{variant})$
Round 6 (HCl)					
Y0244-1	1	V	V	S	0.3
Y0244-2	1	L	K	S	
Y0244-3 <sup>a</sup>	1	L	V	S	
Y0244-4	1	I	K	S	0.9

All variants are in the background of Y0192 (Müller *et al.*, 1998a). *n*, indicates the number of clones found with identical DNA sequence. The wild-type (Fab-12, or Y0192) residue is shown at the top of each column, and the sequence position number is indicated according to Kabat *et al.* (1987).  $K_d(\text{Y0192})/K_d(\text{variant})$  indicates the fold increase in binding affinity *versus* the wild-type humanized antibody Y0192 (see Table 6).

<sup>a</sup> One variant contained a spontaneous mutations, S74W.

Table 6. Binding kinetics of anti-VEGF Fab variants at 25 °C

Variant	$k_{on}/10^4$ ( $M^{-1} s^{-1}$ )	$k_{off}/10^{-4}$ ( $s^{-1}$ )	$K_d$ (nM)	$K_d(Y0192)/K_d(\text{variant})$
Y0192 <sup>a</sup>	4.1	1.2	2.9	1
<b>A. Library-derived</b>				
Y0238-1	2.6	0.09	0.4	7.3
Y0238-3	1.3	$\leq 0.04^b$	$\leq 0.3^b$	$\geq 9.4^b$
Y0238-5	0.57	0.08	1.4	2.1
Y0238-7	1.1	$\leq 0.06^b$	$\leq 0.5^b$	$\geq 5.4^b$
Y0238-10	1.2	0.09	0.8	3.8
Y0242-1	3.8	0.86	2.3	1.3
Y0243-1	4.8	0.45	0.9	3.1
Y0244-1	3.0	2.7	9.0	0.3
Y0244-4	5.2	1.7	3.3	0.9
<b>B. Engineered</b>				
Y0268-1	4.0	0.15	0.38	7.6
Y0313-1	3.5	$\leq 0.05^b$	$\leq 0.15^b$	$\geq 20^b$
Y0192(T28D)	6.8	1.4	2.0	1.4
Y0192(N31H)	4.8	0.37	0.8	3.6
Y0192(H97Y)	2.5	0.045	0.2	14
Y0192(S100aT)	6.8	1.0	1.5	1.9
Y0317	3.6	$\leq 0.05^b$	$\leq 0.14^b$	$\geq 20^b$

Kinetic constants were determined from measurements using a BIAcore™ 2000 instrument with a biosensor chip containing immobilized VEGF(109). Measurements were performed at 25 °C. Fab concentrations were calculated from quantitative amino acid analysis. The equilibrium dissociation constant,  $K_d$ , is calculated from the ratio of the rate constants,  $k_{off}/k_{on}$ . The relative affinity, reported as  $K_d(Y0192)/K_d(\text{variant})$  indicates the fold increase in binding affinity versus the wild-type humanized antibody Y0192. Errors in  $K_d$  were approximately  $\pm 25\%$ . Variant Y0242-1 corresponds to the point mutations Y53W in CDR-H2 of Fab Y0192; for descriptions of the other variants, see Tables 2, 3, 4, 5, and 8.

<sup>a</sup> Data for Y0192 is from Muller *et al.* (1998a).

<sup>b</sup> In some cases, the dissociation rate constant observed was at or near the limit of detection; therefore, the reported  $k_{off}$  and  $K_d$  are upper limits, and the relative affinities are an upper limit.

improvements were seen in CDR-H3 variants Y0238-5 and Y0238-10. However, larger improvements (exceeding the limits of measurement (>five-fold to >ninefold)) were observed for the CDR-H3 variants Y0238-1, Y0238-3, and Y0238-7.

All tested variants (in fact all sequenced clones) from the CDR-H3 library contained the mutation H97Y. In the higher affinity group, Gly was conserved at position 100, while the lower affinity variant contained Ala (known to cause 1.8-fold reduction in Y0192 binding; Muller *et al.*, 1998a) or Asn (Table 4). The S100a position, while quite varied among sequenced clones, was changed to Thr in the higher affinity CDR-H3 variants, and Thr or Lys in the lower affinity ones. Substitutions at Y99, though mostly confined to basic or aromatic residues, apparently had little effect since Y0238-1 (representing the consensus CDR-H3 sequence with Y99R) was not significantly different in affinity from Y0238-3, which retained Y99.

#### Affinity improvements from combinations of CDR mutations

To improve affinity further, several combinations of the phage-selected CDR-H1, H2, and H3 mutations were made by site-directed mutagenesis (Table 7). Among these, the highest affinity was obtained with pY0313-1 (i.e. pY0192 with mutations CDR-H1 (T28D/N31H/I34M) and CDR-H3 (H97Y/S100aT); note I34M is a reversion to Fab-12 wild-type). From BIAcore™ kinetics measurements carried out at 25 °C, this Fab variant had  $\geq 20$ -fold higher affinity than Y0192 (Table 6).

Addition of the Y53W mutation, which alone produced little or no improvement over Y0192, to Y0313-1 (producing variant Y0268-1) actually reduced binding affinity by >twofold (Table 6).

The final Fab version was constructed by removing the phage-expression enhancing mutations in CDR-L1 from pY0313-1 by site-directed mutagen-

Table 7. Anti-VEGF CDR combination variants

Y0192: Variant	CDR-L1					CDR-H1			CDR-H2	CDR-H3	
	R 24	N 26	E 27	Q 28	L 29	T 28	N 31	I 34	Y 53	H 97	S 100a
Y0313-1	-	-	-	-	-	D	H	M	-	Y	T
Y0268-1	-	-	-	-	-	D	H	M	W	Y	T
Y0317	S	S	Q	D	I	D	H	M	-	Y	T
Fab-12	S	S	Q	D	I	-	-	-	-	-	-

Substitutions are shown relative to Y0192. Fab-12 also contains T221 in the heavy chain. Dashes (-) indicate no substitution. Numbering is according to Kabat *et al.* (1987) for both the light chain (CDR-L1) and heavy chain (CDR-H1, H2, H3).

esis. The M4L substitution was identified during phage-humanization experiments (Baca *et al.*, 1997), and the Leu residue was retained so as to preclude possible oxidation of the Met side-chain. The first libraries were constructed from a Fab-12 phagemid derivative, pY0101, which contained a buried framework mutation, V<sub>L</sub>(M4L), as well as a mutation (T221L) at the junction to g3p. Thus the final version, Y0317 (Table 7 and Figure 1) differs from Fab-12 by the following six mutations: V<sub>L</sub>(M4L), V<sub>H</sub>(T28D/N31H/H97Y/S100aT/T221L).

Each of the CDR mutations in H1 and H3 was tested for its effect on VEGF binding affinity by introducing the corresponding point mutation into the parental Y0192 Fab and measuring binding kinetics. The results (Table 6) show a 14-fold and 3.6-fold improvement with substitution of H97Y or N31H, respectively, into the parental Fab. However, T28D or S100aT had identical affinity to Y0192, within experimental error.

We compared Fab-12 and Y0317 Fab affinities in a solution binding assay, using VEGF competition with [<sup>125</sup>I]VEGF for binding to Fab. The results showed Fab-12 having  $K_d^{25^\circ} = 433$  pM and Y0317 Fab having  $K_d^{25^\circ} = 20$  pM, a 22-fold improvement in binding affinity (Figure 2).

Because dissociation kinetics in surface plasmon resonance (SPR) experiments exceeded instrumental capabilities at 25 °C, and in order to assess binding affinity under more physiological conditions, we compared binding affinities of the original humanized antibody Fab-12 with the final variant Y0317 in kinetics experiments at 37 °C.  $k_{on}$  and  $k_{off}$  were faster for both antibodies than at 25 °C, and  $k_{off}$  was clearly measurable above background. Using either immobilized VEGF(109) or immobilized VEGF(165), Y0317 was 120-fold to 140-fold improved in affinity over Fab-12, with a  $K_d^{37^\circ}$  of 80–190 pM (Table 8).

### VEGF Ala-scan of the Y0317 binding epitope

In order to understand how mutations in the Fab affected binding affinity to VEGF, we also tested VEGF variants for binding to the affinity-improved antibody. For these experiments, we made use of the full-length IgG forms of Fab-12 (known as rhuMab VEGF) and Y0317 (termed Y0317-IgG) produced in CHO cells (V. Chisholm,

unpublished results). These VEGF variants were previously used for mapping the parental antibody's binding site on VEGF (Muller *et al.*, 1998a).

In this assay, carried out at 37 °C, VEGF competed with biotin-VEGF with an IC<sub>50</sub> of 9 nM in binding rhuMab VEGF, compared with an IC<sub>50</sub> of 1 nM for Y0317-IgG (Table 9). SPR measurements have shown similar affinity improvement of Y0317-IgG over rhuMab VEGF (H. Lowman, unpublished results).

Alanine mutations of VEGF that affected rhuMab VEGF binding also affected Y0317-IgG. For example, M81A, G88A, and G92A all caused large (100 to >500-fold) losses in binding affinity. And smaller reductions (3 to 30-fold) in binding affinity for both antibodies were seen for I80A, K84A, I91A, E93A, and M94A.

However, significant differences in the magnitude of the effect were observed at certain sites, including Y45A, fourfold reduced in affinity for rhuMab VEGF versus 26-fold for Y0317-IgG; Q89A, 19-fold versus sixfold; and M94A, 11-fold versus 25-fold. Most surprisingly, two mutations that led to loss of detectable binding affinity for rhuMab VEGF (>500-fold) had only modest effects (four- to ninefold) on binding to Y0317-IgG. These differences might suggest a shift in the binding epitope of the antibody, and this possibility was addressed with receptor-inhibition assays and structural analysis, both described below.

### Inhibition of VEGF activity

Cell-proliferation assays have been described (Fairbrother *et al.*, 1998) for the measurement of VEGF mitogenic activity on human umbilical vein endothelial cells. Here, we compared the potency of Fab-12 and the affinity-improved variants Y0238-3 and Y0313-1.

The results (Figure 3) show both variants Y0238-3 and Y0313-1 inhibit VEGF activity more potently than Y0192 Fab. Comparing the Fab forms, variant Y0313-1 appeared at least 30-fold to 100-fold more potent than the wild-type Fab. In additional experiments, Y0317 activity was similar to that of Y0313-1 (data not shown). It should be noted that the amount of VEGF (0.2 nM) used in this assay is potentially limiting for determination of an accurate IC<sub>50</sub> for the mutant. For example, if the bind-

Table 8. Binding kinetics of anti-VEGF Fab variants at 37 °C

Variant	Immobilized	$k_{on}/10^4$ (M <sup>-1</sup> s <sup>-1</sup> )	$k_{off}/10^{-4}$ (s <sup>-1</sup> )	$K_d$ (nM)	$K_d(\text{Fab-12})/K_d(\text{variant})$
Fab-12	VEGF(109)	5.1	6.6	13 ± 2.2	1
Y0317	VEGF(109)	5.4	0.059	0.11 ± 0.02	120
Fab-12	VEGF(165)	5.5	11	20 ± 3.8	1
Y0317	VEGF(165)	5.3	0.074	0.14 ± 0.05	140

Kinetic constants were determined by injecting Fab solutions onto a BIAcore™-2000 instrument with a biosensor chip containing approximately 190 RU of immobilized VEGF(109) or VEGF(165), as indicated. The equilibrium dissociation constant,  $K_d$ , is calculated from the ratio of the rate constants,  $k_{off}/k_{on}$ . The relative affinity, reported as  $K_d(\text{Fab-12})/K_d(\text{variant})$  indicates the fold increase in binding affinity versus the original humanized antibody (Fab-12; Presta *et al.*, 1997) under the specified conditions.

## Light chain:

	1	10	20	30	40	50
Fab-12	DIQMTQSPSSLSASVGDRTTITCSASQDISNYLNWYQKPGKAPKVLIIYF					
Y0192	DIQLTQSPSSLSASVGDRTTITCRANEQLSNYLNWYQKPGKAPKVLIIYF					
Y0317	DIQLTQSPSSLSASVGDRTTITCSASQDISNYLNWYQKPGKAPKVLIIYF					
	1	10	20	30	40	50
		60	70	80	90	100
Fab-12	TSSLHSGVPSRFSGSGSGTDFLTITSSLPEDFATYYCQYSTVPWTFGQ					
Y0192	TSSLHSGVPSRFSGSGSGTDFLTITSSLPEDFATYYCQYSTVPWTFGQ					
Y0317	TSSLHSGVPSRFSGSGSGTDFLTITSSLPEDFATYYCQYSTVPWTFGQ					
		60	70	80	90	100
		110	120	130	140	150
Fab-12	GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV					
Y0192	GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV					
Y0317	GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV					
		110	120	130	140	150
		160	170	180	190	200
Fab-12	DNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQG					
Y0192	DNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQG					
Y0317	DNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQG					
		160	170	180	190	200
		210				
Fab-12	LSSPVTKSFNRGEC					
Y0192	LSSPVTKSFNRGEC					
Y0317	LSSPVTKSFNRGEC					
		210				

Figure 1 (legend shown opposite)

ing affinity ( $K_d$ ) of the mutant is in fact  $<0.2$  nM, then the  $IC_{50}$  in this experiment will appear higher than under conditions of lower VEGF concentration. The result therefore supports the conclusion that the affinity-improved variant is at least 30-fold improved in affinity for VEGF, and that it effectively blocks VEGF activity *in vitro*.

### Structure of the complex

In order to compare the structure and binding site of the affinity-improved antibody with that of

the parental antibody, we determined the complex structure by X-ray crystallography. Crystals of the complex between the receptor binding domain of VEGF (residues 8 to 109) and the affinity-matured Fab Y0317 were grown as described (see Materials and Methods) and diffracted to a maximum resolution of 2.4 Å. The structure was refined starting from the coordinates of the complex between VEGF and the parent of Fab Y0317, Fab-12 (Muller *et al.*, 1998a), and refined to an  $R$ -value of 19.9% ( $R_{free} = 27.4\%$ ) for the reflections between 20 Å and 2.4 Å resolution.

## Heavy chain:

	1	10	20	30	40	50
Fab-12	EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGW					
Y0192	EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGINWVRQAPGKGLEWVGW					
Y0317	EVQLVESGGGLVQPGGSLRLSCAASGY <u>DT</u> FTNYGMNWVRQAPGKGLEWVGW					
	1	10	20	30	40	50
		60	70	80	90	100
Fab-12	INTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYP					
Y0192	INTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYP					
Y0317	<u>INTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYP</u>					
	a	60	70	80	abc	90 96
		110	120	130	140	150
Fab-12	HYYGSSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC					
Y0192	HYYGSSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC					
Y0317	<u>YYYGTS</u> HWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC					
	100	abc	def	110	120	130 140
		160	170	180	190	200
Fab-12	LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG					
Y0192	LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG					
Y0317	LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG					
	150	160	170	180	190	
	210	220	230			
Fab-12	TQTYICNVNHKPSNTKVDKKVEPKSCDKTHT					
Y0192	TQTYICNVNHKPSNTKVDKKVEPKSCDKTHL					
Y0317	TQTYICNVNHKPSNTKVDKKVEPKSCDKTH <u>L</u>					
	200	210	220			

Figure 1. Sequence alignment of the original humanized antibody (Fab-12; Presta *et al.*, 1997), the phage-displayed antibody (Y0192; Muller *et al.*, 1998a) and the affinity-improved antibody (Y0317). Sequential numbering of each chain is shown above the sequences; numbering according to Kabat *et al.* (1987) is shown below. CDR regions are underlined. Positions at which Y0317 differs from Fab-12 are indicated with double underlining.

The final model consists of two Fab fragments bound to the symmetrical poles of the VEGF dimer. Only residues 14-107 of each VEGF monomer are well defined in the electron density, and therefore the six N-terminal and the two C-terminal residues of each monomer were omitted from the model. Each Fab light chain comprises residues 1 to 213, with the C-terminal residue disordered,

whereas for each heavy chain residues 138 to 143 as well as the six C-terminal residues are absent from the model. As in the parental Fab complex, two out of 1050 residues, namely T51 in the V<sub>L</sub> chain of each Fab fragment, are located in the "disallowed regions" (Laskowski *et al.*, 1993) of the Ramachandran plot; 85 % of all residues have their main-chain torsion angles in the "most favored"

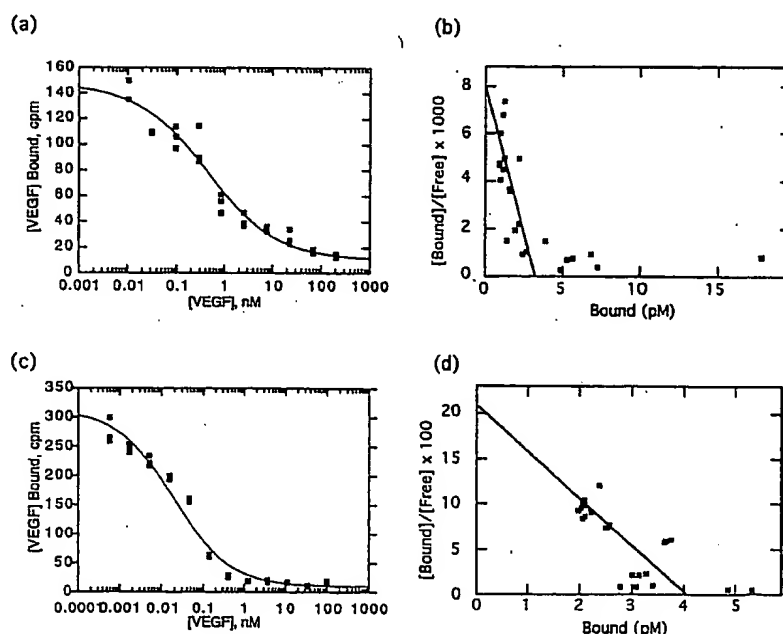


Figure 2. Radiolabeled VEGF binding assay. [ $^{125}$ I]VEGF was equilibrated (23°C) with serial dilutions of unlabeled VEGF and (a) Fab-12 or (c) Y0317. Fabs were captured with an anti-Fab antibody-coated immunosorbant plate. Scatchard analysis (Munson & Rodbard, 1980) with a 1:1 binding model was used to calculate  $K_d$  of (b) 433 ( $\pm 116$ ) pM for Fab-12 and (d) 19.8 ( $\pm 4.3$ ) pM for Y0317.

regions. The average  $B$ -factor of the model is 51.8 Å<sup>2</sup> and the mobility of the individual domains follows the pattern that was previously observed in the crystal structure of VEGF in complex with the Fab-12, with the constant domain dimer (C<sub>L</sub>:C<sub>H1</sub>) of one of the Fabs poorly ordered (Muller *et al.*, 1998a).

Comparison of the final model with that of the parental Fab-VEGF complex (Muller *et al.*, 1998a) shows that the bound structures are very similar overall (Figure 4(a)) with Y0317 binding to the same site on VEGF as Fab-12 (Figure 4(b)). Side-chains show excellent overlap, and the main-chain structures show very little difference. The most prominent difference in contact residues is at H97Y (Figure 4(c); discussed below), where the tyrosine side-chain packs more favorably with VEGF and a buried water molecule from the parental Fab-VEGF complex is absent in the Y0317-Fab-VEGF complex.

## Discussion

### Antibody binding selections and affinity improvement

Here we made use of results from alanine-scanning and the previous structure of a humanized antibody-antigen complex to design Fab-phage libraries that randomized the three heavy-chain CDRs as well as one framework region (FR-H3) for improving the binding affinity of an anti-VEGF antibody. Affinity-improved Fab variants were obtained, with the largest effects seen in variants from the CDR-H3 library, although significant improvement was also obtained from mutation of CDR-H1. We therefore combined two mutations from H1 with two from H3, generating a further improved variant, Y0317. By making point mutations, we showed that the 20-fold (Figure 2)

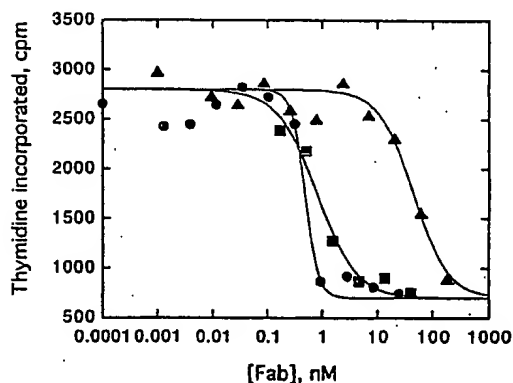


Figure 3. Human umbilical vein endothelial cell (HuVEC) assay of VEGF inhibition. Cells were cultured in the presence of 0.2 nM VEGF and serial dilutions of Fab Y0192 (triangles), Y0238-3 (squares), or Y0313-1 (circles). Cell proliferation was measured by incorporation of [ $^3$ H]thymidine. Curves show four-parameter fits to the data. Each point represents the mean of three treated wells.



Table 9. Alanine scan of VEGF by ELISA at 37 °C

VEGF(109) variant	IC <sub>50</sub> (variant)/IC <sub>50</sub> (VEGF)	
	Fab12-IgG	Y0317-IgG
VEGF(109)	1	1
F17A	1	1
Y21A	1	1
Y45A	4	26
K48A	2	1
Q79A	1	3
I80A	4	5
M81A	>500	930
R82A	>500	4
I83A	>500	9
K84A	3	10
H86A	1	1
Q87A	1	1
G88A	105	87
Q89A	19	6
H90A	1	1
I91A	2	6
G92A	>500	>900
E93A	4	7
M94A	11	25

ELISA assays were carried out using the full-length IgG form of Fab-12 or the IgG form of Y0317 and VEGF(109). Incubation of antibody with VEGF was at 37 °C for five hours. The IC<sub>50</sub> for inhibition by each Ala mutant was evaluated using a four-parameter equation, and the relative affinities calculated as IC<sub>50</sub>(mutant-VEGF)/IC<sub>50</sub>(wild-type VEGF). Under these conditions, Fab12-IgG and Y0317-IgG showed IC<sub>50</sub> values of 9 nM and 1 nM, respectively.

to >100-fold (Table 8) affinity improvement in Y0317 can be attributed to two CDR mutations: H97Y and N31H. In fact, H97Y alone improves binding affinity 14-fold.

Despite the relatively slow  $k_{on}$  and slow  $k_{off}$  of the parental antibody, binding selections described here yielded slower dissociation rates and improved equilibrium dissociation constants. Results of SPR measurements demonstrated that affinity is enhanced mainly through a slower dissociation rate (as opposed to faster association). These results are consistent with the idea of off-rate selection (Hawkins *et al.*, 1992) and with the progressively increased stringency in washing procedures used here (see Materials and Methods and Table 1). Previous binding-optimization efforts have also often yielded larger improvements in  $k_{off}$  than in  $k_{on}$  (see Lowman & Wells, 1993; Yang *et al.*, 1995; Schier *et al.*, 1996). This may suggest fundamental limitations to the improvements in  $k_{on}$  for a given binding site. Even if no conformational changes need occur between free and bound states, the on-rate is limited by the size of the binding interface and the translational and rotational diffusion rates of the binding components (reviewed by Delisi, 1983).

The association rate constants ( $k_{on}$ ) for both the wild-type Y0192 and the final Y0317 antibodies are relatively slow (about  $4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  for both) compared to other antibodies of equal or weaker antigen binding affinity. In fact, the fastest  $k_{on}$  identified for any mutant was  $6.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$

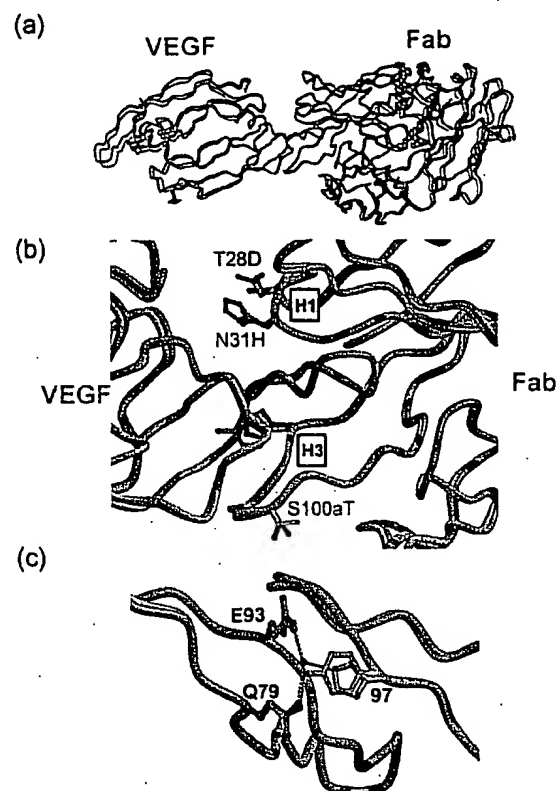


Figure 4. Structure of the affinity-improved Y0317 Fab in complex with VEGF. A superposition of the structure (Muller *et al.*, 1998a) of wild-type humanized antibody Fab-12 (gray) in complex with VEGF (gray) is shown with that of Fab Y0317 (green) in complex with VEGF (yellow). (a) Overall view of the complex, including one Fab molecule bound to one dimer of VEGF (a second Fab molecule is bound at left in the crystal) shows that the binding site for both antibody variants centers on the "80's loop" of VEGF. (b) A view of the four CDR changes between Fab-12 and Y0317 Fab shows that the new D28 and T100a side-chains do not directly contact antigen. However, H31 and Y97 form new contacts. (c) Interactions of H97 and an associated, buried water molecule in the Fab-12 complex, compared with those of Y97 in the Y0317 complex.

(Table 6). Typically,  $k_{on}$  for antibodies binding to protein antigens, including affinity-matured antibodies, has fallen in the range of  $3 \times 10^4$  to  $1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  (Karlsson *et al.*, 1991; Malmberg *et al.*, 1992; Barbas *et al.*, 1994; Yang *et al.*, 1995; Schier *et al.*, 1996; Wu *et al.*, 1998). In this particular protein-protein interaction, a likely explanation for the slow  $k_{on}$  is the high degree of flexibility associated with the binding site both on the Fab and on VEGF. In fact, crystallographic evidence suggests that the "80's loop" region is quite mobile (Muller *et al.*, 1997; Muller *et al.*, 1998b). We are pursuing other strategies to assess whether improvements to  $k_{on}$  can be obtained.

The contributions of point mutations in proteins to the free energy of binding or activation are often additive (Wells, 1990). This principle has been used to produce a variety of affinity-improved protein variants based on point or grouped mutations identified by phage display (Lowman & Wells, 1993; Yang *et al.*, 1995) or point-mutant screening (Wu *et al.*, 1998). Considering the initial library selectants Y0238-3 (>ninefold improved in affinity) and Y0243-1 (3.1-fold improved), we would have predicted an improvement of >27-fold for Y0313-1 or Y0317 (Table 7). In fact, a 22-fold improvement is observed (Figure 2) at 25°C. Addition of the CDR-H1 mutation would be predicted to improve affinity slightly (1.3-fold), but in fact this mutation reduced affinity >twofold (Y0268-1 *versus* Y0313-1; Table 6). Certainly additivity does not always apply, particularly if interacting residues are involved (Wells, 1990). In this case, non-additivity probably results from steric interference between the new Trp in CDR-H2 and the new Tyr in CDR-H3.

To test the energetics of binding by the final Y0317 antibody to VEGF, we made use of a panel of alanine mutants that had been previously constructed for mapping the binding site of the original antibody (Muller *et al.*, 1998a). For these experiments, we made use of the full-length IgG forms of both antibodies. In view of the slow dissociation kinetics for both antibodies, ELISA assays were carried out at 37°C with incubation for at least five hours to insure that equilibrium was reached. Under these conditions two dramatic differences appear in the Ala-scan of VEGF with respect to Y0317 *versus* Fab-12: both R82A and I83A have small effects on binding in Y0317, but result in large decreases in binding for Fab-12. The reasons for these differences are not clear, but R82 and I83 do have significant surface area (55 Å<sup>2</sup> and 32 Å<sup>2</sup>, respectively) buried on binding to VEGF, and make contacts that include residues S100a of CDR-H3 and N52 of CDR-H2 in the wild-type antibody (Muller *et al.*, 1998a).

### Structural analysis of the affinity-matured Fab

The structures of a number of antibodies derived from *in vivo* immunization and hybridoma techniques have been determined, in complex with their antigens (reviewed by Nezlin, 1998), and recently, crystallization and preliminary X-ray studies of a chain-shuffled anti-lysozyme scFv antibody in complex with antigen were reported (Küttner *et al.*, 1998). However, to our knowledge, the Y0317 Fab:VEGF structure is the first report of an *in vitro* affinity-matured Fab in complex with antigen. The structural basis of binding affinity improvement is therefore of interest.

The Fab fragment of the affinity-matured anti-VEGF antibody Y0317 preserves the structure of the original humanized antibody, Fab-12: Superposition with Fab-12 results in an rmsd of only 0.38 Å for a total of 431 C $\alpha$ -positions, demonstrat-

ing the absence of major structural changes between the two molecules. With a total of 1800 Å<sup>2</sup> of solvent-accessible surface buried in each VEGF-Fab interface, the contact area is about 50 Å<sup>2</sup> larger than in the Fab-12 complex. This small increase in buried surface area is mostly due to the exchange of H97 to a tyrosine residue. In the VEGF:Fab-12 complex, H97 buries a solvent-accessible area of 56 Å<sup>2</sup>, while the larger tyrosine side-chain of the matured antibody accounts for 86 Å<sup>2</sup> of buried surface. The tyrosine side-chain also affects the hydrogen-bonding pattern and the number of ordered water molecules in the vicinity. In the parental antibody complex, a water molecule near H97 forms two hydrogen bonds to the side-chains of Q79 and E93 of VEGF (Figure 4(c)). In the complex with the affinity-matured Fab, this water molecule is replaced by the hydroxyl group of the newly introduced tyrosine side-chain at position 97. The H97Y mutation therefore not only increases the amount of buried surface area, but also introduces two additional hydrogen bonds between the ligand and Fab-0317 (Figure 4(c)). This is in good agreement with the observation that this single substitution improves VEGF binding affinity by 14-fold (Table 6). We therefore conclude that this single substitution is responsible for the majority of the improvement in binding affinity of Y0317 compared to the parent antibody.

In contrast, despite the availability of the crystal structures of both complexes, it remains uncertain what the structural basis is of the 3.6-fold enhanced binding caused by the N31H mutation. The side-chains of the asparagine and the histidine residues in this position adopt identical conformations in both crystal structures, and the amount of buried surface is not significantly increased in the VEGF:Fab-Y0317 complex. The only difference we can detect is a slight possible improvement in the hydrophobic interactions between the histidine side-chain and the phenyl group of VEGF residue F17, which has rotated slightly compared to the parent complex. It is unclear whether this could contribute to the increased affinity.

Neither of the remaining differences between Fab-12 and Fab-Y0317 has a significant effect on the binding affinity towards VEGF, and the structures show that these residues contribute only marginally to the interface. Some interactions are present between VEGF and the main-chain atoms of the serine and threonine residues in position 100a of the two Fabs, but the side-chains of these residues are not in contact with VEGF. Finally, no contact exist between VEGF and T28 (or D28) of the Fab fragments (the closest point on VEGF to this residue is more than 6 Å distant).

In summary, the analysis and comparison of the two crystal structures are in very good agreement with the results of the binding assays on the various single mutants of the Fab fragments. Although it is not possible to quantify the effects introduced by the amino acid exchanges solely based on the crystal structures, the detailed crystallographic

analysis supports and enables us to interpret the binding data.

### Biological implications for antibody inhibition of VEGF

An inhibitory antibody of improved affinity may have improved potency or efficacy in treating diseases associated with VEGF expression. Preceding versions of the anti-VEGF antibody described here, including the murine A4.6.1 (Kim *et al.*, 1993), the humanized version Fab-12 (Presta *et al.*, 1997), as well as Y0192 (Muller *et al.*, 1998a), clearly demonstrated sufficient affinity to effect inhibition of VEGF activity. Here, we show that an affinity-improved variant, Fab Y0317, can inhibit endothelial cell proliferation *in vitro* with least 30-fold greater potency than the parental humanized Fab (Figure 3).

We have limited our optimization strategy to a subset of heavy-chain CDR residues implicated by alanine-scanning and crystallography (Muller *et al.*, 1998a). Furthermore, not all combinations of phage-derived mutations have been tested. One may therefore reasonably ask whether Y0317, with  $K_d^{25} = 20$  pM and  $K_d^{37} = 130$  pM, is the globally optimum variant for binding to this particular epitope (or others) on VEGF. Other affinity optimization efforts have resulted in protein-protein binding affinities in the low picomolar range, from  $K_d = 6$  pM to 15 pM (see, e.g. Lowman & Wells, 1993; Schier *et al.*, 1996; Yang *et al.*, 1995). Indeed, we cannot exclude the possibility that higher affinity variants of the A4.6.1 antibody could be produced. However, it seems unlikely that further affinity improvement would greatly enhance biological potency or efficacy because for effective inhibition, the antibody must certainly occupy a significant fraction (perhaps >99%) of the available (VEGF) binding sites. Serum VEGF concentrations of about 20 pM in normals, and of >300 pM in patients with metastatic carcinoma, have been observed (Kraft *et al.*, 1999). Local or effective concentrations are likely higher. If we conservatively assume the effective concentration of VEGF *in vivo* to be about 400 pM, then 400 pM of even an infinite-affinity Fab would be required to block all sites.

Other factors may limit the improvement in potency of a full-length IgG resulting from an improvement in intrinsic binding affinity of the Fab for antigen. The full-length IgG form of the antibody may benefit from an avidity effect *in vivo*, especially since VEGF is known to associate with proteoglycans on the cell surface (Gitay-Goren *et al.*, 1992). Even in cell-based assays, the IgG form of Fab-12 is a more effective inhibitor than the Fab form (data not shown). Finally, the half-life for dissociation of the affinity-improved antibody is already significant, even on the time-scale of the half-life of clearance for IgG's (days to weeks). The effect of an improved association rate constant for antibody in this system is unknown.

The fact that point (Ala) mutations in the antibody binding site on VEGF sometimes have lesser effects on the binding of Y0317 than on the binding of Fab-12 may suggest that the optimized binding site is more tolerant than the parental one of variations in the antigen. Indeed, Y0317 showed greatly enhanced affinity for murine VEGF over that of Fab-12 (data not shown), though still >100-fold weaker than its affinity for human VEGF. This could provide an advantage against naturally arising VEGF variants.

## Materials and Methods

### Construction of phage libraries and mutagenesis

A variant of the Fab-12 antibody (a humanized form of murine antibody A4.6.1) was previously identified from phage-displayed Fab libraries for improved expression on phage particles (Muller *et al.*, 1998a). We made use of the plasmid pY0192, a phagemid construct with ampicillin (or carbenicillin) resistance, as the parental ("wild-type") construct for libraries described here. To prevent contamination by wild-type sequence (Lowman *et al.*, 1991; Lowman, 1998), templates with the TAA stop codon at each residue targeted for randomization were prepared from CJ236 *E. coli* cells (Kunkel *et al.*, 1991). Libraries are designated according to the mutagenic oligonucleotides used for their construction: YC265, TCC TGT GCA GCT TCT GGC NNS NNS TTC NNS NNS NNS GGT ATG AAC TGG GTC CG, randomizing residues 27-28, 30-32 in CDR-H1; YC266, GAA TGG GTT GGA TGG ATT AAC NNS NNS NNS GGT NNS CCG ACC TAT GCT CCG G, randomizing residues 52a-54, 56 in CDR-H2; YC103, GAA TGG GTT GGA TGG ATT NNS NNS NNS NNS GGT GAA CCG ACC TAT G, randomizing residues 52-54 in CDR-H2; YC81, C TGT GCA AAG TAC CCG NNS TAT NNS NNS NNS CAC TGG TAT TTC GAC, randomizing residues 97, 99-100b in CDR-H3; and YC101, CGT TTC ACT TTT TCT NNS GAC NNS TCC AAA NNS ACA GCA TAC CTG CAG, randomizing residues 71, 73, and 76 in the "FR-H3" region. An additional library in CDR-H2 was designed to insert three new residues: YC90, GATGG ATT AAC ACC TAT NNS NNS NNS ACC GGT GAA CCG ACC.

The products of random mutagenesis reactions were electroporated into XL1-Blue *E. coli* cells (Stratagene) and amplified by growing 15-16 hours with M13KO7 helper phage. The complexity of each library, ranging from  $2 \times 10^7$  to  $1.5 \times 10^8$ , was estimated based on plating of the initial transformation onto LB plates containing carbenicillin.

Site-directed mutagenesis for point mutations was carried out as above, using appropriate codons to produce the respective mutations, and sequences were confirmed by single-strand DNA sequencing using Sequenase<sup>TM</sup> (USB).

### Phage binding selections

For each round of selection, approximately  $10^9$ - $10^{10}$  phage were screened for binding to plates (Nunc Maxi-sorp 96-well) coated with 2 µg/ml VEGF(109) in 50 mM carbonate buffer (pH 9.6) and blocked with 5% (w/v) instant milk in 50 mM carbonate buffer, (pH 9.6). Also included were phage prepared from a non-displaying

control phagemid (pCAT), which confers chloramphenicol resistance, as a means of measuring background and enrichment (Lowman & Wells, 1993). Bound phage were eluted with 0.1 M HCl and immediately neutralized with one-third volume of 1 M Tris (pH 8.0). The eluted phage were propagated by infecting XL1 cells for the next selection cycle as described (Lowman, 1998).

In the first cycle, the VEGF plate was incubated with Fab-phage, then was briefly washed to remove bound phage. In the second cycle, binding and washing were followed by a one hour dissociative incubation at room temperature with binding buffer, after which the plate was again washed prior to acid elution. This process was repeated in rounds 3, 4 and 5, except that 1  $\mu$ M VEGF was included in the dissociative incubation, and the incubation time was increased to 2, 18, and 37 hours, respectively. During these selections, Y0192 phage showed enrichments ranging from 1.5-fold (at the lowest stringency) to 22,000-fold (using a two hour dissociation incubation). However, further increases in stringency (rounds 4-5) resulted in decreasing enrichments for the control phage, with higher enrichments observed for certain libraries, especially the two CDR-H2 libraries and the CDR-H3 library (Table 1).

In cycle 6, a 17 hour dissociative incubation at room temperature was followed by an additional 30 hour incubation at 37°C (also including VEGF in the buffer). Under these conditions, Y0192-phage showed only slight binding enrichment (20-fold), whereas the CDR-H3 library phage were enriched by 3500-fold. Cycle 7 was carried out with a 63 hour dissociative incubation, after which only small enrichment factors were observed. However, some libraries were continued through eight cycles (with 120 hours of dissociative incubation in the presence of VEGF), after which Fab-phage were still recoverable by acid elution (data not shown).

### Purification of Fab

For small-scale preparations, Y0317 Fab and mutants were prepared from *E. coli* shake-flasks as described (Muller *et al.*, 1998a).

For large-scale preparation, whole cell broth was obtained from a ten liter *E. coli* fermentation. The cells were lysed with a Manton-Gaulin homogenizer (two passes at 6000 psi; lysate temperature maintained at 15-25°C with a heat exchanger). A 5% (v/v) solution of polyethylene imine (PEI), pH 6.0, was added to the lysate to give a final concentration of 0.25% (v/v). The lysate was mixed for 30 minutes at room temperature. The suspension was centrifuged, and the supernatant (containing the Fab) was processed further. The pH of the supernatant was adjusted to 6.0 with 6 M HCl, followed by dilution to a conductivity of 5 mS/cm with purified water. The conditioned supernatant was loaded onto a BakerBond ABx ion-exchange column. Following a wash with the column equilibration buffer, the Fab was eluted with an increasing sodium chloride gradient in the equilibration buffer. Fractions containing the Fab were identified by SDS-PAGE. The BakerBond ABx column fractions were pooled, pH adjusted to 5.5 with 1 M Mes and diluted to a conductivity of 5 mS/cm with purified water. The conditioned BakerBond ABx pool was loaded onto a SP Sepharose HP cation exchange column (Pharmacia). Once again, the Fab was eluted with a sodium chloride-containing gradient. Fractions containing the Fab were identified by SDS-PAGE. The level of

purity of Fab (as determined by SDS-PAGE) after this two column purification was >95%.

### BIAcore™ binding analysis

The VEGF-binding affinities of Fab fragments were calculated from association and dissociation rate constants measured using a BIAcore™-2000 surface plasmon resonance system (BIAcore, Inc., Piscataway, NJ). A biosensor chip was activated for covalent coupling of VEGF using *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) according to the supplier's (BIAcore, Inc., Piscataway, NJ) instructions. VEGF(109) or VEGF(165) was buffer-exchanged into 20 mM sodium acetate, pH 4.8 and diluted to approximately 50  $\mu$ g/ml. Aliquots of VEGF were injected at a flow rate of 2  $\mu$ l/minute to achieve approximately 700-1400 response units (RU) of coupled protein. A solution of 1 M ethanolamine was injected as a blocking agent.

For kinetics measurements, twofold serial dilutions of Fab were injected in PBS/Tween buffer (0.05% Tween-20 in phosphate-buffered saline) at 25°C or 37°C at a flow rate of 10  $\mu$ l/minute. Equilibrium dissociation constants,  $K_d$  values from SPR measurements were calculated as  $k_{off}/k_{on}$  (Tables 6 and 8).

### Radiolabeled VEGF binding assay

Solution binding affinity of Fabs for VEGF was measured by equilibrating Fab with a minimal concentration of [<sup>125</sup>I]-labeled VEGF(109) in the presence of a titration series of unlabeled VEGF, then capturing bound VEGF with an anti-Fab antibody-coated plate.

To establish conditions for the assay, microtiter plates (Dynex) were coated overnight with 5  $\mu$ g/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23°C). In a non-adsorbant plate (Nunc #269620), 100 pM or 26 pM [<sup>125</sup>I]VEGF(109) was mixed with serial dilutions of Fab-12 or Fab Y0317, respectively. Fab-12 was incubated overnight; however, the Fab Y0317 incubation was continued for 65 hours to insure that equilibrium was reached. Thereafter, the mixtures were transferred to the capture plate for incubation at room temperature for one hour. The solution was then removed and the plate washed eight times with 0.1% Tween-20 in PBS. When the plates had dried, 150  $\mu$ l/well of scintillant (Micro-Scint-20; Packard) was added, and the plates were counted on a Topcount gamma counter (Packard) for ten minutes. Concentrations of each Fab were chosen to give  $\leq 20\%$  of maximal binding.

For competitive binding assays, Dynex plates were coated and blocked as above, and serial threefold dilutions of unlabeled VEGF(109) were made in PBS/Tween buffer in a Nunc plate. [<sup>125</sup>I]VEGF(109) was added, followed by addition of a fixed concentration of Fab-12 or Fab Y0317. The final concentrations of Fab-12, and Fab Y0317 were 100 pM and 10 pM, respectively. After incubation (as above), bound VEGF was captured and quantified as described above. The binding data was analyzed using a computer program to perform Scatchard analysis (Munson & Rodbard, 1980) for determination of the dissociation binding constants,  $K_d$ , for Fab-12 and Fab Y0317.

### ELISA assay of VEGF Ala mutants

The binding affinities of VEGF Ala mutants for full-length Fab-12-IgG (known as rhuMab VEGF) and Y0317-IgG, a full-length IgG form of the improved antibody expressed in CHO cells (V. Chisholm, unpublished results) were measured as previously described (Muller *et al.*, 1997; Muller *et al.*, 1998a) for the murine antibody A4.6.1, except that the temperature was increased to 37°C, and the incubation time increased to five hours, to insure that equilibrium was reached with the high-affinity antibody.

### Cell-based assay of VEGF inhibition

Several versions of the anti-VEGF antibody were tested for their ability to antagonize VEGF(165) induction of the growth of HuVECs (human umbilical vein endothelial cells). The 96-well plates were seeded with 1000 HuVECs per well and fasted in assay medium (F12:DMEM 50:50 supplemented with 1.5% (v/v) dialyzed fetal bovine serum) for 24 hours.

The concentration of VEGF used for inducing the cells was determined by first titrating to identify the amount of VEGF that can stimulate 80% of maximal DNA synthesis. Fresh assay medium containing fixed amounts of VEGF (0.2 nM final concentration), and increasing concentrations of anti-VEGF Fab or mab were then added. After 40 hours of incubation, DNA synthesis was measured by incorporation of tritiated thymidine. Cells were pulsed with 0.5 µCi per well of [<sup>3</sup>H]thymidine for 24 hours and harvested for counting, using a TopCount gamma counter.

### Crystallization and refinement

The complex between the Fab fragment of affinity-matured, humanized antibody Y0317 Fab and the receptor binding fragment of VEGF (VEGF(109)) was purified and crystallized as described for the analogous complex with the parental humanized Fab-12 fragment (Muller *et al.*, 1998a). The resulting crystals had symmetry consistent with space group *P*<sub>2</sub><sub>1</sub>, with cell parameters *a* = 89.1 Å, *b* = 66.4 Å, *c* = 138.7 Å, and β = 94.7°, and were isomorphous with the crystals obtained with the

parent complex. A data set was collected from a single frozen crystal at beam line 5.0.2 at the Advanced Light Source, Berkeley, and processed using programs MOSFLM and SCALA (CCP4, 1994). The final data set (*R*<sub>merge</sub> = 7.3%) is described in Table 10. Starting with the model of Brookhaven Protein Data Bank entry 1bj1 (Muller *et al.*, 1998a), the structure was refined using the programs X-PLOR (Brünger *et al.*, 1987) and REFMAC (CCP4, 1994). The free *R*-value was monitored using the identical set of reflections sequestered before refinement of parent complex. The differences in the primary structure between Fab-12 and Fab-Y0317 were modeled using the program O (Jones *et al.*, 1991). After correction for anisotropy and application of a bulk solvent correction, the *R*-value reached its final value of 19.9% for all reflections greater than 0.2σ (see Table 10; *R*<sub>free</sub> = 27.4%).

### Protein Data Bank accession number

The coordinates for the VEGF:Y0317 Fab complex have been deposited in the Protein Data Bank, accession number 1cz8.

### Acknowledgments

We thank Lyn Deguzman and Tom Zioncheck for providing [<sup>125</sup>I]VEGF; Alan Padua and Bill Henzel for quantitative amino acid analysis; James Bourell for mass spectrometry; Vanessa Chisholm and Lynne Krummen for construction of cell lines; and Manuel Baca, Napoleone Ferrara, Yves Muller, Leonard Presta, and James Wells for many helpful discussions.

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Table 10. Crystallographic data and refinement statistics

A. Data collection	Overall	Last shell
Resolution range (Å)	30-2.4	2.53-2.40
No. of observations	208,257	22,278
Unique reflections	61,742	8900
Completeness (%)	97.4	96.7
Mean <i>I</i> /σ( <i>I</i> )	13.6	2.7
<i>R</i> <sub>sym</sub>	0.073	0.38
B. Refinement		
Resolution range (Å)	20-2.4	
No. of reflections	61,689	
No. of atoms	8577	
rmsd bond lengths (Å)	0.013	
rmsd angles (deg.)	1.9	
rmsd improper angles (deg.)	0.92	
rmsd <i>B</i> -factors for all bonded atoms, Å <sup>2</sup>	3.5	
Number of main-chain torsion angles in disallowed regions of Ramachandran plot <sup>a</sup>	2	

<sup>a</sup> See Laskowski *et al.* (1993).

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Edited by I. A. Wilson

(Received 19 July 1999; received in revised form 7 September 1999; accepted 13 September 1999)



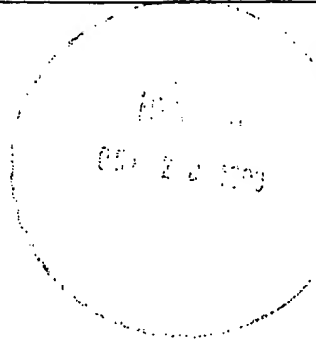
DEPARTMENT OF HEALTH & HUMAN SERVICES

**rhFab VEGF**

Food and Drug Administration  
1401 Rockville Pike  
Rockville MD 20852-1448

Our Reference: BB-IND 8633

Genentech, Incorporated  
Attention: Robert L. Garnick, Ph.D.  
Vice President, Regulatory Affairs  
1 DNA Way  
South San Francisco, CA 94080-4990



OCT 13 1999

Dear Dr. Garnick:

The Center for Biologics Evaluation and Research has received your **Investigational New Drug Application (IND)**. The following product name and BB-IND number have been assigned to this application. They serve only to identify it and do not imply that this Center either endorses or does not endorse your application.

**BB-IND #: 8633**

**SPONSOR: Genentech, Incorporated**

**PRODUCT NAME: Humanized Monoclonal Antibody Fragment (rhFab V2)  
(E. coli, Genentech) to Vascular Endothelial Growth Factor  
(VEGF), Intravitreal**

**DATE OF SUBMISSION: October 6, 1999**

**DATE OF RECEIPT: October 7, 1999**

This BB-IND number should be used to identify all future correspondence and submissions, as well as telephone inquiries concerning this IND. Please provide an **original and two copies of every submission to this file**. Please include three originals of all illustrations which do not reproduce well.

It is understood that studies in humans will not be initiated until 30 days after the date of receipt shown above. If this office notifies you, verbally or in writing, of serious deficiencies that require correction before human studies can begin, it is understood that you will continue to withhold such studies until you are notified that the material you have submitted to correct the deficiencies is satisfactory. If such a clinical hold is placed on this file, you will be notified in writing of the reasons for placing the IND on hold.

10-18-99 P02:54 IN  
10-18-99 P:



You are responsible for compliance with applicable portions of the Public Health Service Act, the Federal Food, Drug, and Cosmetic Act, and the Code of Federal Regulations (CFR). A copy of 21 CFR Part 312, pertaining to INDs, is enclosed. Copies of other pertinent regulations are available from this Center upon request. The following points regarding obligations of an IND sponsor are included for your information only, and are not intended to be comprehensive.

Progress reports are required at intervals not exceeding one year and are due within 60 days of the anniversary of the date that the IND went into effect [21 CFR 312.33]. Any unexpected, fatal or immediately life-threatening reaction associated with use of this product must be reported to this Division by telephone or facsimile transmission no later than seven calendar days after initial receipt of the information, and all serious, unexpected adverse experiences must be reported, in writing, to this Division and to all study centers within fifteen calendar days after initial receipt of this information [21 CFR 312.32].

Charging for an investigational product in a clinical trial under an IND is not permitted without the prior written approval of the FDA.

Prior to use of each new lot of the investigational biologic in clinical trials, please submit the lot number, the results of all tests performed on the lot, and the specifications when established (i.e., the range of acceptable results).

If not included in your submission, please provide copies of the consent forms for each clinical study. A copy of the requirements for and elements of informed consent are enclosed. Also, please provide documentation of the institutional review board approval(s) for each clinical study.

All laboratory or animal studies intended to support the safety of this product should be conducted in compliance with the regulations for "Good Laboratory Practice for Nonclinical Laboratory Studies" (21 CFR Part 58, copies available upon request). If such studies have not been conducted in compliance with these regulations, please provide a statement describing in detail all differences between the practices used and those required in the regulations.

Item 7a of form FDA 1571 requests that either an "environmental assessment," or a "claim for categorical exclusion" from the requirements for environmental assessment, be included in the IND. If you did not include a response to this item with your application, please submit one. See the enclosed information sheet for additional information on how these requirements may be addressed.

Sponsors of INDs for products used to treat life-threatening or severely debilitating diseases are encouraged to consider the interim rule outlined in 21 CFR 312.80 through 312.88.

Telephone inquiries concerning this IND should be made directly to me at (301) 827-5101. Correspondence regarding this file should be addressed as follows:

Center for Biologics Evaluation and Research  
Attn: Office of Therapeutics Research and Review  
HFM-99, Room 200N  
1401 Rockville Pike  
Rockville, MD 20852-1448

If we have any comments after we have reviewed this submission, we will contact you.

Sincerely yours,

A handwritten signature in black ink that reads "Kay Schneider". The signature is written in a cursive, slightly slanted style.

Kay Schneider, M.S.  
Consumer Safety Officer  
Division of Application Review and Policy  
Office of Therapeutics  
Research and Review  
Center for Biologics  
Evaluation and Research

Enclosures (3): 21 CFR Part 312  
21 CFR 50.20, 50.25  
Information sheet on 21 CFR 25.24



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration  
Rockville, MD 20852

JAN 27 2006

Genentech, Inc.  
Attention: Robert L. Garnick, Ph.D.  
Senior Vice President, Regulatory Affairs, Quality, and Compliance  
1 DNA Way  
South San Francisco, CA 94080-4990

Dear Dr. Garnick:

We have received your biologics license application (BLA) submitted under section 351 of the Public Health Service Act for the following biological product:

**Our Submission Tracking Number (STN):** BL #125156/0

**Name of Biological Product:** Lucentis™ (ranibizumab)

**Indication:** Treatment for patients with neovascular age-related macular degeneration

**Date of Application:** December 29, 2005

**Date of Receipt:** December 30, 2005

**User Fee Goal Date:** June 30, 2006

All applications for new active ingredients, new dosage forms, new indications, new routes of administration, and new dosing regimens are required to contain an assessment of the safety and effectiveness of the product in pediatric patients unless this requirement is waived or deferred. We note that you have not fulfilled the requirement. We are waiving the requirement for pediatric studies for this application.

If you have not already done so, promptly submit the *content of labeling* (21 CFR 601.14(b)) in electronic format as described at the following website:  
<http://www.fda.gov/oc/datacouncil/spl.html>.

We will notify you within 60 days of the receipt date if the application is sufficiently complete to permit a substantive review.

We request that you submit all future correspondence, supporting data, or labeling relating to this application in triplicate, citing the above STN number. Please refer to <http://www.fda.gov/cder/biologics/default.htm> for important information regarding therapeutic biological products, including the addresses for submissions. Effective August 29, 2005, the new address for all submissions to this application is:

Food and Drug Administration  
Center for Drug Evaluation and Research  
Therapeutic Biological Products Document Room  
5901-B Ammendale Road  
Beltsville, MD 20705-1266

If you have any questions, please contact the Regulatory Project Manager, Lori Gorski, at (301) 796-0722.

Sincerely,

A handwritten signature in black ink, appearing to read "Maureen P. Dillon-Parker", with a stylized flourish at the end.

Maureen P. Dillon-Parker  
Chief, Project Management Staff  
Division of Anti-Infective and  
Ophthalmology Products  
Office of Antimicrobials  
Center for Drug Evaluation and Research



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration  
Rockville, MD 20852

BLA 125156

MAR 14 2006

Genentech, Inc.  
Attention: Robert L. Garnick, Ph.D.  
Senior Vice President, Regulatory Affairs, Quality & Compliance  
1 DNA Way  
South San Francisco, California 94080-4990

Dear Dr. Garnick:

This letter is in regard to your biologics license application (BLA) submitted under section 351 of the Public Health Service Act.

We have completed an initial review of your application dated December 29, 2005, for Lucentis (ranibizumab injection) to determine its acceptability for filing. Under 21 CFR 601.2(a), your application was filed on February 28, 2006. The user fee goal date is June 30, 2006. This acknowledgment of filing does not mean that we have issued a license nor does it represent any evaluation of the adequacy of the data submitted.

At this time, we have not identified any potential review issues. Our filing review is only a preliminary review, and deficiencies may be identified during substantive review of your application. Following a review of the application, we shall advise you in writing of any action we have taken and request additional information if needed.

Please refer to <http://www.fda.gov/cder/biologics/default.htm> for important information regarding therapeutic biological products, including the addresses for submissions.

Please use the following address for any amendments to your application:

Food and Drug Administration  
Center for Drug Evaluation and Research  
Therapeutic Biological Products Document Room  
5901-B Armmendale Road  
Beltsville, MD 20705-1266

If you have any questions, call Lori M. Gorski, Project Manager, at (301) 796-0722.

Sincerely,

Maureen Dillon Parker  
Chief, Project Management Staff  
Division of Anti-Infective and Ophthalmology Products  
Office of Antimicrobial Products  
Center for Drug Evaluation and Research

# Fax



Division of Anti-Infective and Ophthalmology  
Products, HFD-520  
Center for Drug Evaluation and Research  
5901-B Ammendale Road  
Beltsville, MD 20705-1266

To: Nick Bruno

From: Lori Gorski, Project Manager

Fax: 650-467-3198

Fax: 301-796-0722

Phone: 650-225-5017

Phone: 301-796-9882

Pages: 2 (including cover page)

Date: 3/15/06

Re: 74 day letter for BLA 125156

☐ Urgent ☐ For Review ☐ Please Comment ☐ Please Reply ☐ Please Recycle

THIS DOCUMENT IS INTENDED ONLY FOR THE USE OF THE PARTY TO WHOM IT IS ADDRESSED AND MAY CONTAIN INFORMATION THAT IS PRIVILEGED, CONFIDENTIAL AND PROTECTED FROM DISCLOSURE UNDER APPLICABLE LAW. If you are not the addressee, or a person authorized to deliver the document to the addressee, you are hereby notified that any review, disclosure, dissemination or other action based on the content of the communication is not authorized. If you have received this document in error, please immediately notify us by telephone and return it to us at the above address by mail. Thank you.

Nick -

Per our conversation yesterday. Please contact  
me if you have questions

Thanks

Lori Gorski

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent of: Manuel Baca *et al.* – § 156

Patent No.: 6,884,879

Issued: April 26, 2005

Application No: 08/908,469

Docket No: 22338-80060

Assignee: Genentech, Inc.

Unit: OPLA; Attn: K. Ferriter

For: ANTI-VEGF ANTIBODIES – Application for § 156 Patent Term Extension

Mail Stop: **Patent Ext.**

Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

**POWER OF ATTORNEY BY ASSIGNEE**

The assignee of the entire right, title, and interest in U.S. Patent No. 6,884,879 (granted on application serial no. 08/908,469), Genentech, Inc., hereby appoints the practitioners associated with

**CUSTOMER NUMBER 33694**

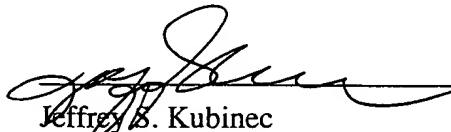
as its attorneys and agents to prosecute the captioned patent/application, and to transact all business in the U.S. Patent and Trademark Office connected therewith.

Pursuant to 37 C.F.R. § 3.73(b), the undersigned states that Genentech, Inc. is the assignee of the entire right, title, and interest in the captioned patent/application by virtue of an assignment by the inventors to Genentech Inc. recorded at Reel 008872/ Frame 0429.

The undersigned, whose title is supplied below, is authorized to act on behalf of the assignee.

Respectfully submitted,

GENENTECH, INC.



Jeffrey S. Kubinec

Associate General Counsel – Patent Law

*JK* 08-15-06

Date



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent of: Manuel Baca *et al.* -- § 156

Docket No: 22338-80060

Patent No.: 6,884,879

Assignee: Genentech, Inc.

Issued: April 26, 2005

Unit: OPLA


Application No: 08/908,469

For: ANTI-VEGF ANTIBODIES – Application for  
§ 156 Patent Term Extension

CERTIFICATE OF MAILING - 37 C.F.R. § 1.10  
EXPRESS MAIL LABEL NO. ER 736 919 908 US

Mail Stop Patent Ext.  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

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Signature

YVONNE T. REYES  
Printed Name

Aug 25 2006  
Date

APPLICATION FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. § 156

Dear Sir:

Applicant, Genentech, Inc., hereby submits this application for extension of the term of United States Letters Patent 6,884,879 under 35 U.S.C. § 156 by providing the following information in accordance with the requirements specified in 37 C.F.R. § 1.740.

Applicant represents that it is the assignee of the entire interest in and to United States Letters Patent No. 6,884,879, granted to Manuel Baca; James A. Wells; Leonard G. Presta; Henry B. Lowman; and Yvonne Man-yea Chen (Baca *et al.*) by virtue of an assignment of such patent to Genentech, Inc., recorded December 29, 1997, at Reel 8872, Frame 0429.

1. Identification of the Approved Product [§ 1.740(a)(1)]

The name of the approved product is LUCENTIS™. The name of the active ingredient of LUCENTIS™ is ranibizumab. Ranibizumab is a recombinant humanized monoclonal IgG<sub>1</sub> antibody antigen-binding fragment (Fab) based on a humanized framework with complementarity-determining regions (CDRs) derived from a murine monoclonal antibody that binds to human Vascular Endothelial Growth Factor (VEGF).



**2. Federal Statute Governing Regulatory Approval of the Approved Product  
[§ 1.740(a)(2)]**

The approved product was subject to regulatory review under, *inter alia*, the Public Health Service Act (42 U.S.C. § 201 *et seq.*) and the Federal Food, Drug and Cosmetic Act (21 U.S.C. § 355 *et seq.*).

**3. Date of Approval for Commercial Marketing [§ 1.740(a)(3)]**

LUCENTIS™ was approved for commercial marketing or use under § 351 of the Public Health Service Act on **June 30, 2006**.

**4. Identification of Active Ingredient and Certifications Related to Commercial Marketing of Approved Product [§ 1.740(a)(4)]**

- (a) The active ingredient of LUCENTIS™ is ranibizumab. Ranibizumab is a humanized monoclonal IgG<sub>1</sub> antibody antigen-binding fragment produced by an *E. coli* expression system. It contains human framework regions (FRs) and the complementarity-determining regions (CDRs) derived from a murine antibody that binds to VEGF.
- (b) Applicant certifies that ranibizumab had not been approved for commercial marketing or use under the Federal Food, Drug and Cosmetic Act, the Public Health Service Act or the Virus-Serum-Toxin Act prior to the approval granted on June 30, 2006 to the present Applicant.
- (c) Ranibizumab has been approved for the treatment of patients with neovascular (wet) age-related macular degeneration. *See* LUCENTIS™ product label, provided as Attachment A.
- (d) LUCENTIS™ was approved for commercial marketing pursuant to § 351 of the Public Health Service Act (42 U.S.C. § 262) under Genentech's existing Department of Health and Human Services (DHHS) U.S. License No. 1048. *See* LUCENTIS™ approval letter, provided as Attachment B.

**5. Statement Regarding Timeliness of Submission of Patent Term Extension Request [§ 1.740(a)(5)]**

Applicant certifies that this application for patent term extension is being timely submitted within the sixty (60) day period permitted for submission specified in 35 U.S.C. § 156(d)(1) and 37 C.F.R. § 1.720(f). The last date on which this application may be submitted is August 28, 2006.

**6. Complete Identification of the Patent for Which Extension Is Being Sought [§ 1.740(a)(6)]**

The complete identification of the patent for which an extension is being sought is as follows:

- (a) Names of the inventors: Manuel Baca; James A. Wells; Leonard G. Presta; Henry B. Lowman; and Yvonne Man-yee Chen.
- (b) Patent Number: 6,884,879 (“the ‘879 patent”)
- (c) Date of Issue: April 26, 2005
- (d) Date of Expiration: August 6, 2017<sup>1</sup>

**7. Copy of the Patent for Which an Extension is Being Sought [§ 1.740(a)(7)]**

A copy of U.S. Patent No. 6,884,879 is provided as Attachment D to the present application.

**8. Copies of Disclaimers, Certificates of Correction, Receipt of Maintenance Fee Payment, or Reexamination Certificate [§ 1.740(a)(8)]**

- (a) U.S. Patent No. 6,884,879 is not subject to a terminal disclaimer.
- (b) A Certificate of Correction was issued for U.S. Patent No. 6,884,879 on August 23, 2005. A request for a second Certificate of Correction was filed on May 19, 2006. The request asks that the related application data in the patent be amended to accurately reflect that application No. 08/883,504 had been converted to provisional application no. 60/126,446. A copy of the Certificate of Correction as well as the Request for the second Certificate of Correction is provided in Attachment E to the present application.
- (c) The first maintenance fee for U.S. Patent No. 6,884,879 will be due on October 26, 2008.

---

<sup>1</sup> The face sheet of the ‘879 patent notes that a claim of priority is made to U.S. Serial No. 08/833,504 (“the ‘504 application”). Prior to abandonment of that application and issuance of the ‘879 patent, a petition was filed to convert the ‘504 application to a provisional application. The petition was submitted on April 6, 1998 and granted thereafter (a copy of the PTO’s entry of the petition is being provided for convenience as Attachment C) and the ‘504 application became U.S. Provisional Application No. 60/126,446. Accordingly, the filing date of the 08/908,469 application, from which the ‘879 patent issued, is used in calculating the expiration date of the ‘879 patent.

(d) U.S. Patent No. 6,884,879 has not been the subject of a reexamination proceeding.

**9. Statement Regarding Patent Claims Relative to Approved Product [§ 1.740(a)(9)]**

*The statements below are made solely to comply with the requirements of 37 C.F.R. § 1.740(a)(9). Applicant notes that, as the M.P.E.P. acknowledges, § 1.740(a)(9) does not require an applicant to show whether or how the listed claims would be infringed, and that this question cannot be answered without specific knowledge concerning acts performed by third parties. As such, these comments are not an assertion or an admission of Applicant as to the scope of the listed claims, or whether or how any of the listed claims would be infringed, literally or under the doctrine of equivalents, by the manufacture, use, sale, offer for sale or the importation of any product.*

- (a) At least claims 4 and 5 of U.S. Patent No. 6,884,879 claim the active pharmaceutical ingredient in the approved product or a method that may be used to make or use that ingredient.
- (b) Pursuant to M.P.E.P. § 2753 and 37 C.F.R. § 1.740(a)(9), the following explanation is provided which shows how the above-listed claims of the '879 patent claim a method of making the approved product.

*(1) Description of the approved product and its method of production*

The approved product is described in Section 11 of the approved label for LUCENTIS™ as follows, a copy of which is provided as Attachment A.

LUCENTIS™ (ranibizumab injection) is a recombinant humanized IgG1 kappa isotype monoclonal antibody fragment designed for intraocular use. Ranibizumab binds to and inhibits the biologic activity of human vascular endothelial growth factor A (VEGF-A). Ranibizumab has a molecular weight of approximately 48 kilodaltons and is produced by an *E. coli* expression system in a nutrient medium containing the antibiotic tetracycline. Tetracycline is not detectable in the final product.

LUCENTIS™ is a sterile, colorless to pale yellow solution in a single-use glass vial. LUCENTIS™ is supplied as a preservative-free, sterile solution in a single-use glass vial designed to deliver 0.05 mL of 10 mg/mL LUCENTIS™ aqueous solution with 10 mM histidine HCL, 10% α, α-trehalose dihydrate, 0.01% polysorbate 20, pH 5.5.

Ranibizumab is further characterized in a scientific reference, Chen *et al*, published in 1999 in the Journal of Molecular Biology (JMB) entitled "Selection and Analysis of an Optimized Anti-VEGF Antibody: Crystal Structure of an

Affinity-matured Fab in Complex with Antigen.”<sup>2</sup> For example, the heavy chain sequence of ranibizumab, designated as Y0317 in Chen *et al.*, is displayed in Figure 1. The CDRH1, 2, and 3 of ranibizumab are underlined in the figure and have the following sequences:

CDRH1 contains 10 amino acids of the sequence: GYDFTHYGMN (which using the three-letter designation for each amino acid is the sequence Gly Tyr Asp Phe Thr His Tyr Gly Met Asn):

CDRH2 contains 17 amino acids of the sequence: WINTYTGEPTYAADFKR (which using the three-letter designation for each amino acid is the sequence Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe Lys Arg); and

CDRH3 contains 14 amino acids of the sequence: YPYYYGTSHWYFDV (which using the three-letter designation for each amino acid is the sequence Tyr Pro Tyr Tyr Tyr Gly Thr Ser His Trp Tyr Phe Asp Val)

The article also discusses the lineage of the ranibizumab antibody fragment. In that respect, the article states that “[a] murine monoclonal antibody, A.4.6.1, was found to block VEGF-dependant cell proliferation *in vitro* and to antagonize tumor growth *in vivo*. [Citation omitted]. The murine mAb was previously humanized in Fab form to yield a variant known as Fab-12.” [Citation omitted] See p. 866, left col., ¶1. The abstract explains that the authors affinity-matured Fab-12 and obtained Fab fragment Y0317, now known as ranibizumab. According to the article, ranibizumab was derived from the humanization and affinity-maturation of a non-human, murine monoclonal antibody that binds to VEGF.

(2) *Claim 4 and 5*

Claim 4 of the '879 patent reads as follows:

4. A process of producing a humanized anti-VEGF antibody comprising culturing the host cell of claim 3 so that the nucleic acid is expressed.
5. The process of claim 4 further comprising recovering the humanized anti-VEGF antibody from the host cell culture.

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<sup>2</sup> 293:865-881 (1999) (Attachment F)

Claim 4 depends from claim 3, which in turn depends from claim 2, which depends from claim 1. Therefore, claim 4 contains the limitations of claims 1, 2, and 3. Claim 5 further depends from claim 4. Claims 1, 2, and 3 of the '879 patent read as follows:

1. Isolated nucleic acid encoding a humanized variant of a parent anti-VEGF antibody which parent antibody comprises non-human variable domains, wherein said humanized variant binds human VEGF and comprises the following heavy chain Complementary Determining Region (CDR) amino acid sequences: SEQ ID NO:128 as CDRH1, SEQ ID NO:2 as CDRH2 and SEQ ID NO:129 as CDRH3.
2. A vector comprising the nucleic acid of claim 1.
3. An isolated host cell comprising the vector of claim 2.

*Comparison of a nucleic acid encoding ranibizumab to the limitations of claim 1*

Claim 1 pertains to an isolated nucleic acid encoding a humanized variant of a parent anti-VEGF antibody, which parent antibody comprises non-human variable domains, wherein said humanized variant binds human VEGF and comprises the recited CDRs.

As discuss above, ranibizumab was initially produced by the humanization and affinity-maturation of a murine, anti-VEGF monoclonal antibody and Fab fragment. The parental murine monoclonal antibody and its humanized fragment, by definition, contain non-human variable domains. Applicant also notes that the term "antibody" as defined in the '879 patent specification includes, in addition to full-length antibodies, antibody fragments such as Fab, Fab', F(ab)<sub>2</sub> and Fv so long as those fragments retain the desired biological activity, i.e., binding to VEGF (*See, e.g.*, Col 8, lines 38-49). Ranibizumab, being an Fab fragment that binds VEGF, falls within the scope of the term antibody as used in Claim 1. Moreover, as demonstrated by the following comparison, the CDR sequences disclosed in SEQ ID NOS:128, 2 and 129 of the '879 patent are identical to, or read on, the CDR sequence of ranibizumab disclosed in the Chen *et al* reference describe above.

SEQ ID NO:128 (CDRH1)	Gly Tyr Xaa <sup>3</sup> Phe Thr Xaa Tyr Gly Met Asn
Ranibizumab CDRH1 (Chen <i>et al</i> )	Gly Tyr Asp Phe Thr His Tyr Gly Met Asn
SEQ ID NO:2 (CDRH2)	Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe Lys Arg
Ranibizumab CDRH2 (Chen <i>et al</i> )	Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe Lys Arg
SEQ ID NO:129 (CDRH3)	Tyr Pro Xaa <sup>4</sup> Tyr Tyr Gly Xaa Ser His Trp Tyr Phe Asp Val
Ranibizumab CDRH3 (Chen <i>et al</i> )	Tyr Pro Tyr Tyr Tyr Gly Thr Ser His Trp Tyr Phe Asp Val

The CDR sequences recited in claim 1 of the '879 patent thus read on the CDR sequences of ranibizumab.

*Comparison of the production of ranibizumab to the limitations of claim 2, 3 and 4*

Claim 2 and 3 of the '879 patent recite a vector containing the nucleic acid of claim 1 and an isolated host cell containing said vector, respectively. Claim 4 additionally requires a method of producing an anti-VEGF antibody. Pursuant to the label, ranibizumab is produced using a recombinant *E. coli* cell line expression system. Such an expression system employs a *vector*, which as explained above would contain a nucleic acid covered by claim 1, and an isolated host cell, in this case *E. coli*, containing the vector. Using the vector of claim 2 in an *E. coli* host cell of claim 3 as a method of producing ranibizumab, would fall within the scope of claim 4 of the '879 patent.

*Comparison of the production of ranibizumab to Claim 5*

Claim 5 pertains to a process wherein the humanized anti-VEGF antibody is recovered from the host cell culture used to make the antibody. Applicant asserts that claim 5 covers a method of making the approved product for at least the following reasons.

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<sup>3</sup> SEQ ID NO:128 notes that the third amino acid can be either Thr or Asp. The sixth amino acid in SEQ ID NO:128, on the other hand, is not limited to any particular amino acid.

<sup>4</sup> SEQ ID NO:129 notes that the third amino acid can be either Tyr or His. Similarly, it notes that the seventh amino acid can be either Ser or Thr.

According to the label, Lucentis™ contains ranibizumab in a form that is sterile and suitable for intraocular injection. *See* Label ¶11. The label also states that ranibizumab is produced using an *E. coli* expression system. While the label does not provide the details of how ranibizumab is purified for use in Lucentis™, the antibody would necessarily have to be separated and recovered from the cell culture in which it was produced in order for it to be used in a sterile, injectible therapeutic. Accordingly, the purification steps used in the manufacture of ranibizumab are covered by claim 5.

**10. Relevant Dates Under 35 U.S.C. § 156 for Determination of Applicable Regulatory Review Period [§ 1.740(a)(10)]**

*(a) Patent Issue Date*

U.S. Patent No. 6,884,879 was issued on April 26, 2005.

*(b) IND Effective Date [35 U.S.C. § 156(g)(1)(B)(i); 37 C.F.R. § 1.740(a)(10)(i)(A)]*

The date that an exemption under § 505(i) of the Federal Food, Drug and Cosmetic Act became effective (*i.e.*, the date that an investigational new drug application (“IND”) became effective) for LUCENTIS™ (referred to as “Humanized Monoclonal Antibody Fragment (rhuFab V2)(E. coli, Genentech) to Vascular Endothelial Growth Factor (VEGF), Intravitreal”) was October 7, 1999. The IND was assigned number BB-IND # 8633. A copy of the letter from the FDA reflecting the effective date of the IND is provided in Attachment G. The application date for this IND was October 6, 1999.

*(c) BLA Submission Date [35 U.S.C. § 156(g)(1)(B)(i); 37 C.F.R. § 1.740(a)(10)(i)(B)]*

The BLA was submitted by Genentech to the FDA on December 29, 2005. The BLA was assigned number BL# 125156/0. A copy of the letter from the FDA acknowledging receipt of the BLA and reflecting the BLA submission date is provided in Attachment H.

*(d) BLA Issue Date [35 U.S.C. § 156(g)(1)(B)(ii); 37 C.F.R. § 1.740(a)(10)(i)(C)]*

The FDA approved biologic license application 125156/0 authorizing the marketing of LUCENTIS™ on June 30, 2006. LUCENTIS™ was approved under Department of Health and Human Services (DHHS) U.S. License No. 1048. A copy of the approval letter from the FDA is provided as Attachment B.



**11. Summary of Significant Events During Regulatory Review Period [§ 1.740(a)(11)]**

Pursuant to 37 C.F.R. § 1.740(a)(11), the following provides a brief description of the activities of Genentech, Inc. before the FDA in relation to the regulatory review of LUCENTIS™. The brief description lists the significant events that occurred during the regulatory review period for the approved product. In several instances, communications to or from the FDA are referenced. Pursuant to 37 C.F.R. § 1.740(a)(11), 21 C.F.R. § 60.20(a), and M.P.E.P. § 2753, copies of all such communications are not provided in this application, but can be obtained from records maintained by the FDA.

- On October 6, 1999, Genentech submitted to FDA (*See* Attachment G) an investigational new drug application for a recombinant humanized monoclonal antibody fragment (rhuFab V2, now known as Ranibizumab) against Vascular Endothelial Growth Factor (VEGF). The antibody was developed as a potential new therapeutic in treating patients with the exudative (wet or neovascular) form of age-related macular degeneration (AMD).
- On October 7, 1999 FDA made BB-IND #8633 effective via a communication mailed to Genentech on October 13, 1999 (*See* Attachment G). According to the FDA, initiation of trials could begin 30 days after October 7, 1999.
- The first human clinical trial (Phase I) was initiated on February 8, 2000 followed by Phase II human trials and Phase III human trials, some of which remain ongoing at the time of this application.
- On February 5, 2002, representatives of Genentech and the FDA (CBER and CDER) participated in a Type C meeting to discuss the proposed clinical development plan for ranibizumab in AMD.
- On October 31, 2002 representatives of Genentech and FDA (CBER and CDER) participated in an Type B End-of-Phase II meeting.
- Beginning in approximately March 2003, and continuing at the time of this application, Phase III studies have been conducted. The three Phase III trials forming the basis of the Biologics License Application (BLA), FVF2598g, FVF2587g, and FVF3192g are studies of two year duration with primary endpoints of one year. FVF2587g and FVF3192g, along with extension study FVF3426g and safety study FVF3689g, remain ongoing at the time of this application.
- On September 21, 2005 representatives of Genentech and CDER participated in a Type B Pre-BLA submission meeting to discuss information requirements for the BLA.

- Genentech submitted a BLA for ranibizumab for the treatment of patients with wet AMD on December 29, 2005 (*See Attachment H*).
- FDA acknowledged receipt of the BLA for ranibizumab via a communication mailed to Genentech dated January 27, 2006. The letter indicated that FDA had assigned the Submission Tracking Number (STN) of BL #125156/0 to the BLA (*See Attachment H*).
- By way of a communication mailed to Genentech on March 14, 2006 FDA made Genentech aware that the BLA for ranibizumab was filed on February 28, 2006 and that FDA had assigned a user fee goal date of June 30, 2006 (*See Attachment I*).
- On June 30, 2006 FDA approved BLA 125156/0, issuing marketing authorization for LUCENTIS™ (*See Attachment B*).

**12. Statement Concerning Eligibility for and Duration of Extension Sought Under 35 U.S.C. § 156 [37 C.F.R. § 1.740(a)(12)]**

- (a) In the opinion of the Applicant, U.S. Patent No. 6,884,879 is eligible for an extension under § 156 because:
  - (i) one or more claims of the '879 patent claim the approved product or a method of making or using the approved product;
  - (ii) the term of the '879 patent has not been previously extended on the basis of § 156;
  - (iii) the '879 patent has not expired;
  - (iv) no other patent has been extended pursuant to § 156 on the basis of the regulatory review process associated with the approved product, LUCENTIS™;
  - (v) there is an eligible period of regulatory review by which the patent may be extended pursuant to § 156;
  - (vi) the applicant for marketing approval exercised due diligence within the meaning of § 156(d)(3) during the period of regulatory review;
  - (vii) the present application has been submitted within the 60-day period following the approval date of the approved product, pursuant to § 156(c); and
  - (viii) this application otherwise complies with all requirements of 35 U.S.C. § 156 and applicable rules and procedures.
- (b) The period by which the term of the '879 patent is requested by Applicant to be extended is **307 days**.
- (c) The requested period of extension of term for the '879 patent corresponds to the regulatory review period that is eligible for extension pursuant to § 156, based on the facts and circumstances of the regulatory review associated with the approved product LUCENTIS™ and the issuance of the '879 patent. The period was determined as follows.
  - (i) The relevant dates for calculating the regulatory review period, based on the events discussed in the section above, are the following.

Exemption under FDCA § 505(i) became effective	October 7, 1999
Patent was granted	April 26, 2005
Biologics License Application (BLA) under PHSA § 351 was filed	December 29, 2005
BLA was approved	June 30, 2006

- (ii) The '879 patent was granted during the period specified in § 156(g)(1)(B)(i) (*i.e.*, the period from the date of the grant of the exemption under § 505(i) of the FDCA until the date of submission of the BLA). Pursuant to § 156(b) and (c)(2), the calculated regulatory review period therefore includes a component of time between when the patent was granted and when the BLA was submitted (1/2 of 246 days or 123 days).
- (iii) The patent was granted prior to the start of the period specified in § 156(g)(1)(B)(ii) (*i.e.*, the period from the date of submission of the BLA until the date of approval). The regulatory review period under § 156(b) therefore includes a component equal to the total number of days in that period that are after the BLA was submitted (184 days).
- (iv) The period determined according to § 156(b), (c)(2), and (g)(1) for the approved product (*i.e.*, the number of days following the date of issue of the patent between the dates of submission and of approval of the BLA for LUCENTIS™) is 307 days.
- (v) The '879 patent will expire on August 6, 2017.
- (vi) The date of approval of the approved product is June 30, 2006.
- (vii) The date that is fourteen years from the date of approval of the approved product is June 30, 2020.
- (viii) The period measured from the date the patent expires (*i.e.*, August 6, 2017) until the end of the fourteen-year period specified in § 156 (c)(3) (*i.e.*, June 30, 2020) is approximately 1054 days (*ca.* 2 years, 10 months and 24 days).

- (ix) The number of days in the regulatory review period determined pursuant to § 156(g)(1)(B)(ii) does not exceed the number of days that the patent may be extended pursuant to §156(c)(3). As such, the period by which the patent may be extended is not limited by the fourteen-year rule of §156(c)(3).
- (x) The '879 patent issued after the effective date of Public Law No. 98-417. As such, the two- or three-year limit of 35 U.S.C. § 156(g)(6)(C) does not apply.

**13. Statement Pursuant to 37 C.F.R. § 1.740(a)(13)**

Pursuant to 37 C.F.R. § 1.740(a)(13), Applicant acknowledges its duty to disclose to the Director of the PTO and to the Secretary of Health and Human Services any information which is material to the determination of entitlement to the extension sought, particularly as that duty is defined in 37 C.F.R. § 1.765.

**14. Applicable Fee [§ 1.740(a)(14)]**

Our check in payment of the fee prescribed in 37 C.F.R. § 1.20(j) for a patent term extension application under 35 U.S.C. § 156 accompanies this application. Please deduct any additional required fees from, or credit any overpayments to our deposit account no. 18-1260.

**15. Name and Address for Correspondence [§ 1.740(a)(14)]**

Please direct all inquiries, questions, and communications regarding this application for term extension to:

Jeffrey P. Kushan  
SIDLEY AUSTIN LLP  
1501 K Street, N.W.  
Washington, D.C. 20005  
Phone: 202-736-8914  
Fax: 202-736-8111  
email: jkushan@sidley.com

The correspondence address for U.S. Patent No. 6,884,879 is unchanged for all other purposes. A Power of Attorney granted to the undersigned by the patent assignee, a copy of which is included with this application as Attachment J, accompanies this communication.

Two additional copies of this application are enclosed, in compliance with 37 C.F.R. § 1.740(b).

Sincerely,

A handwritten signature in black ink, appearing to read 'Jeffrey P. Kushan', with a long horizontal flourish extending to the right.

Jeffrey P. Kushan  
Attorney for Applicant  
Registration No. 43,401

Sidley Austin LLP  
1501 K Street, N.W.  
Washington, D.C. 20005

Dated: August 24, 2006

## INDEX OF ATTACHMENTS

- Attachment A: Lucentis™ Product Label
- Attachment B: Lucentis™ Biologics' License Application Approval
- Attachment C: 05/13/99 PTO petition grant
- Attachment D: U.S. Patent No. 6,884,879
- Attachment E: Certificate of Correction and request for a second Certificate of Correction
- Attachment F: Chen *et al.*, "Selection and Analysis of an Optimized Anti-VEGF Antibody: Crystal Structure of an Affinity-Matured Fab in Complex with Antigen." *J. Mol. Bio.*, 293:865-881 (1999).
- Attachment G: 10/13/99 Letter from FDA to Genentech regarding IND acceptance/effective date
- Attachment H: 01/27/06 Letter from the FDA to Genentech regarding receipt and acceptance of BLA Application
- Attachment I: 03/14/06 Letter from the FDA to Genentech regarding 02/28/06 filing of BLA, and 06/30/06 assignation of User Fee Goal Date
- Attachment J: Power of Attorney by Assignee